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FUNCTIONAL INTEGRITY OF NK AND NK-like T LYMPHOCYTES FROM OVARIAN NEOPLASIA PATIENTS

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Orientador: Prof. Dr. Carlos Alberto Petta

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FUNCTIONAL INTEGRITY OF NK AND NK-like T LYMPHOCYTES FROM OVARIAN NEOPLASIA PATIENTS

Dissertação de Mestrado apresentada à Pós-Graduação em Tocoginecologia, da Faculdade de Ciências Médicas da Universidade Estadual de Campinas-UNICAMP para obtenção do Título de Mestre em Ciências da Saúde, área de concentração em Fisiopatologia Ginecológica.

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“Uma vez que reconhecemos nossos limites, vamos além deles.”

Albert Einstein

“A aprendizagem e o conhecimento que temos, é, no máximo pouco, quando comparado à nossa ignorância.”

Platão

SUMÁRIO

LISTA DE ABREVIATURAS

CAISM - Centro de Atenção Integral a Saúde da Mulher

cél./ml – Células por mililitro

CMSP - Células Mononucleares do Sangue Periférico

DMSO - *Dimethyl sulfoxide*

E – *Effector*

FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo

FBS – *Fetal Bovine Serum*

FIGO - *Fédération Internationale de Gynécologie et d'Obstétrique*

FITC - Isotiocianato de Fluoresceína

g – Força Centrífuga Relativa

ºC – Graus Celsius

h – horas

HLA – *Human Leukocyte Antigen*

IL – Interleucina

INF – Interferon

K562 – Linhagem Tumoral Eritromieloblastóide Humana

KIR - *Killer Immunoglubuline-Like Receptors*

LAK - *Lymphokine-activated killer*

LCE – Laboratórios Clínicos Especializados

MAbs – *Monoclonal Antibodies*

MIC - *MHC class I Related Chain Protein*

µl – Microlitro

µM – Micromolar

ml – Mililitro

mm – Milímetro

mM – Milimolar

min – Minutos

NCR - *Natural Citotoxic Receptors*

ng – Nanograma

NK – *Natural Killer*

NK-*like* T – *Natural Killer-like T*

PBMC - *Peripheral blood mononuclear cells*

PBS - *Phosphate-Buffered Saline*

PE – Ficoeritrina

PE-Cy7 - Ficoeritrina / Cianina 7

SAS – *Statistical Analysis Software*

SCGM – *Stem* Cell *Growth Medium*

SEM - *Standard Error Mean*

SFB – Soro Fetal Bovino

SSC/ FSC – *Side Scatter / Foward Scatter*

T – *Target*

TNF – *Tumor Necrosis Factor*

TRAIL - *TNF-Related Apoptosis-Inducing Ligand*

UI – Unidades Internacionais

ULBP - *UL16-Binding Protein*

Unicamp – Universidade Estadual de Campinas

RESUMO

As células NK são linfócitos conhecidos pela capacidade de eliminar uma variedade de células malignas, sem a necessidade de estímulo prévio e por meio de um processo de reconhecimento inato, o qual envolve receptores ativadores e inibidores. Esta capacidade também foi observada nos linfócitos NK-*like* T, porém a via de reconhecimento imunológico envolvida pode ser tanto a inata quanto a adaptativa. Neste estudo, avaliou-se a atividade funcional e a expressão de receptores ativadores DNAM-1, NKp30 e NKp44 das células NK e NK-*like* T, do sangue e ascite de pacientes com neoplasia de ovário. Foi colhido sangue de 24 pacientes com neoplasia de ovário (11 benignas, Bng; 6 malignas sem metástases, Mlg e 7 malignas com metástases, MlgMt) e ascite (Asc) de 6 pacientes, mediante consentimento assinado. Células mononucleares foram separadas por gradiente de centrifugação. A ativação das células NK e NK-*like* T foi determinada pela expressão da molécula CD107a por meio da citometria de fluxo, quando coincubadas com as células K562 (proporção 1:1). Foram comparados os efeitos de dois procedimentos de cultivo celular, um de curta duração (18h) empregando meio RPMI-1640 suplementado com SFB (10%), L-glutamina (2mM) e IL-2 (1000UI/ml) e outro de longa duração (21 dias) empregando meio SCGM CellGro suplementado com anti-CD3 (10ng/ml, nos primeiros 5 dias), IL-2 (1000UI/ml) e SFB (10%) em relação à atividade funcional e a expressão dos receptores ativadores. A atividade funcional das células NK pareceu ser afetada em decorrência da evolução da doença. A estimulação com IL-2 de curta duração aumentou a atividade funcional das células NK, mas não significantemente. A estimulação com IL-2 de longa duração aumentou a atividade funcional das células NK significantemente ($p<0.001$).

A porcentagem de células NK expressando os receptores ativadores DNAM-1, NKp30 e NKp44 aumentou significantemente ($p<0.05$) depois da estimulação com IL-2 de longa duração. As células NK-*like* T apresentaram baixa atividade funcional nos períodos de estimulação de pré e curta duração e nenhuma atividade na estimulação de longa duração. Foi observado que a integridade funcional das células NK foi comprometida com o desenvolvimento do câncer de ovário. A estimulação de longa duração resultou em um maior número de células NK funcionais quando comparado à de curta duração, conferindo este método para uso imunoterápico. A estimulação de longa duração também foi eficiente no aumento da expressão do receptor ativador DNAM-1 nas células NK, sugerindo este método para reverter a supressão deste receptor, que frequentemente é observada em pacientes com carcinoma de ovário. A falta de ativação das células NK-*like* T (pré, curta e longa duração) sugere que, no sistema experimental aplicado, estas células não são ativadas pela via imune inata.

Palavras Chave: Células NK, Células NK-*like* T, neoplasia de ovário, imunoterapia, NCRs (*natural cytotoxic receptors*).

ABSTRACT

NK cells are lymphocytes known by their ability to eliminate a variety of malignant cells without previous stimulation, in a process involving innate recognition by an array of stimulatory and inhibitory receptors. Similarly, the variant subset of NK-like T lymphocytes has been reported to eliminate tumor cells, but the targeting process might involve either innate or adaptive immune recognition. This study evaluated the functional activation of NK and NK-like T cells, the expression of activating receptors DNAM-1, NKp30 and NKp44 (pre, short- and long-term IL-2 stimulated), from blood and ascites of ovarian neoplasia patients. Blood was collected from 24 patients with ovarian neoplasias after signed consent: 11 benign (Bng), 6 malignant without metastasis (Mlg) and 7 malignant with metastasis (MlgMt). Ascites (Asc) was collected from 6 patients with ovarian neoplasia. Mononuclear cells were separated by gradient centrifugation. NK and NK-like T cells activation (pre, short- and long-term stimulated) was evaluated against K562 (1:1 ratio) by the expression of CD107a, analyzed by flow cytometry. Short-term stimulation with IL-2 (1000UI/ml) was conducted overnight in RPMI-1640 medium supplemented with FBS (10%) and L-glutamine (2mM). Long-term stimulation was conducted by a 21 day culturing process with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), IL-2 (1000UI/ml) and FBS (10%). The functional activation of NK cells pre stimulated seemed to be impaired with the progression of the disease. Short-term stimulation increased NK cells activation, but not significantly. Long-term stimulation increased NK cells activation significantly ($p<0.001$). The percentage of NK cells expressing the activating receptors DNAM-1, NKp30 and NKp44 increased significantly ($p<0.05$) after

long-term stimulation. NK-like T cells showed low activation on pre and short-term and none activation on long-term IL-2 stimulation. Long-term stimulation resulted in a much higher number of functional NK cells compared to short-term, entitling this method for adoptive therapy. Long-term stimulation was particularly efficient to up-regulate DNAM-1 activating receptor on NK cells, representing a way to overcome down-regulation demonstrated on patients with ovarian carcinoma. The lack of activation of NK-like T cells (pre, short- and long-term IL-2 stimulated) suggests that, in the method applied; these cells are not activated through innate pathway. **Keywords:** NK cells, NK-like T cells, ovarian neoplasia, immunotherapy, NCRs (natural cytotoxic receptors).

Introdução

1. INTRODUÇÃO

O carcinoma de ovário é o mais letal entre os cânceres ginecológicos, ocupando o quinto lugar entre as causas de morte por câncer nas mulheres norte americanas. A letalidade do carcinoma de ovário é atribuída ao estádio avançado em que a doença, frequentemente, se encontra no momento do diagnóstico. Quando a enfermidade está limitada ao ovário, a cirurgia pode ser curativa, beneficiando 92% das pacientes tratadas. Entretanto, na ocasião do diagnóstico, 63% das pacientes já apresentam comprometimento de outros tecidos além dos ovários e, apesar dos avanços terapêuticos, somente 27% das pacientes em estádio avançado da doença estarão vivas cinco anos após o diagnóstico. [1]

O epitélio responde por 90% dos cânceres de ovário. [2] Um aspecto amplamente conhecido do comportamento do câncer de ovário é a capacidade deste tumor de semear a cavidade peritoneal com células tumorais, inclusive com a formação de ascite. Células tumorais também podem se espalhar através de vasos linfáticos que drenam os ovários para os linfonodos pélvicos e paraórtícos. [3] Atualmente, o tratamento de tumores em grau avançado baseia-se na cirurgia acompanhada pela quimioterapia com agentes derivados de platina (carboplatina e cisplatina) em combinação com taxanos (paclitaxel e docitaxel). [4] Contudo, a maioria das pacientes (60-70%) que recidivam entre 2 e 5 anos após a primeira linha de tratamento, evoluem para óbito. [5] A natureza difusa do câncer de ovário e a rápida disseminação metastática através da cavidade peritoneal limitam a eficiência da terapia tradicional. Há, portanto, a necessidade de novas abordagens terapêuticas visando eliminar células tumorais para prevenir à recidiva.

O uso da imunoterapia celular contra o câncer tem sido investigado desde a introdução das células LAK (*lymphokine-activated killer*) na década de 80. [6, 7] A abordagem mais comum tem sido a infusão intravenosa de células efetoras citotóxicas autólogas ou alogênicas, com o potencial de eliminar células tumorais. As células NK (*natural killers*) tem se destacado entre as populações de células efetoras com potencial antitumoral. [8] O principal obstáculo nos estudos clínicos é que estas células são normalmente encontradas em pequena quantidade entre as células mononucleares do sangue periférico (CMSP) e em preparações de células efetoras, como nas preparações de células LAK. [9]

A possibilidade de se usar as células *natural killer* (NK) para o tratamento de neoplasias malignas humanas tem aumentado nos últimos anos. [10, 11] Isto é consequência do desenvolvimento de novos métodos de isolamento celular ou expansão *ex vivo* que permitem obter células NK humanas em quantidades suficientes para sua utilização em ensaios clínicos [12, 13, 14] e da melhor compreensão de como a interação receptores-ligantes influencia as células NK no sentido de reconhecer e eliminar as células tumorais. Neste sentido, células NK obtidas por métodos de expansão *ex vivo* tem sido avaliadas, principalmente, para o tratamento de neoplasias hematológicas. [8, 15] Todavia, estratégias terapêuticas baseadas no emprego das células NK podem ser particularmente interessantes para o tratamento da doença residual e controle de metástases nas neoplasias epiteliais, como no carcinoma de ovário. A origem epitelial do carcinoma de ovário sugere que as células tumorais expressam ligantes para os receptores ativadores NKG2D e DNAM-1 das células NK, de fato, a presença destes ligantes vem sendo confirmada por estudos recentes. [16, 17, 18, 19, 20] Além disso, alterações da expressão do

HLA de classe I nas células do carcinoma de ovário favorecem a ação citotóxica mediada pelas células NK. [21]

As células NK constituem 5-15% dos linfócitos presentes no sangue periférico humano, sendo identificadas pela expressão do marcador CD56 e ausência do CD3 ($CD3^-$ $CD56^+$). [22] Foram inicialmente identificadas pela capacidade de eliminar uma variedade de células tumorais sem a necessidade de estímulo prévio, [23, 24, 25] entretanto, a estimulação das células NK com interleucina-2 (IL-2), aumenta sua capacidade citotóxica em decorrência do aumento da expressão de receptores associados à ativação celular. [26, 27, 28, 29] Este conhecimento favorece a ideia de se superar a imunossupressão, frequentemente atribuída ao paciente com câncer, empregando-se estratégias *ex vivo* para a expansão e estimulação das células citotóxicas. [30]

A ativação citotóxica das células NK é determinada por meio do reconhecimento inato, pelo balanço de sinais inibidores e ativadores, resultantes da interação dos receptores presentes nas células NK com seus respectivos ligantes sobre a célula alvo. [31, 32] Se ativadas, as células NK desencadeiam o processo de secreção de perforina e granzima-B ou induzem apoptose da célula alvo através dos ligantes FasL e TRAIL (*TNF-related apoptosis-inducing ligand*). As células NK também atuam como células reguladoras da resposta imune inata e ajudam no desenvolvimento da resposta imune adaptativa, através da secreção de citocinas pró-inflamatórias como interferon-γ (INF-γ), fator de necrose tumoral-α (*tumor necrosis factor-α*, TNF-α) e fator estimulador de colônia de granulócitos e macrófagos (*granulocyte macrophage colony-stimulating factor*, GM-CSF). [33, 34, 35]

Diversos receptores inibidores e ativadores têm sido caracterizados nas células NK. [36] Entre os principais receptores inibidores encontram-se: a) receptores KIR (*killer immunoglubuline-like receptors*) de cauda intracelular longa, que reconhecem moléculas do complexo antigênico leucocitário humano (HLA) de classe I dos grupos A, B e C; [37] b) receptor CD94-NKG2A que reconhece o HLA de classe I do grupo E e G. [38] O HLA de classe I é expresso por praticamente todas as células nucleadas de um indivíduo, desempenhando papel fundamental para que o sistema imune reconheça as células do organismo como próprias. Dessa forma, as células NK poupam células autossômicas que expressam níveis normais de HLA de classe I, enquanto eliminam células alteradas, como as células tumorais ou infectadas por vírus que, frequentemente, perdem a expressão do HLA I. [37, 39]

Entre os receptores ativadores destacam-se os receptores citotóxicos naturais (*natural citotoxic receptors*, NCRs) NKp46 e NKp30, expressos constitutivamente nas células NK, e o receptor NKp44, expresso após a ativação das células NK pela interleucina 2 (IL-2). [27, 40, 41, 42, 43] Estes receptores também auxiliam no reconhecimento de células tumorais ou infectadas por vírus. A identidade dos seus ligantes vem sendo descrita recentemente, como por exemple o ligante B7-H6 para o receptor NKp30. [42] A densidade da expressão dos NCRs varia entre indivíduos e correlaciona-se diretamente com a capacidade das células NK de eliminar células alteradas. [44] Outro importante receptor ativador das células NK é o NKG2D cuja expressão também ocorre nos linfócitos T citotóxicos (γ/δ T e α/β T-CD8). O NKG2D reconhece como ligantes moléculas homólogas ao HLA de classe I representadas por proteínas transmembrana como o MIC (*MHC class I related chain protein*, MIC/A e MIC/B) e o ULBP4 (*UL16-biding protein*) e

proteínas ancoradas à superfície celular pelo glicofosfatidilinositol (GPI) como os ULBPs 1, 2 e 3. [45, 46, 47, 48, 49] Em humanos, o aumento da expressão de MIC e ULBP está relacionado a diferentes formas de estresse celular, como a infecção viral e a transformação maligna. [45, 50] De fato, a expressão de MICA/B tem sido observada na maioria dos tumores epiteliais humanos, incluindo carcinomas de mama, ovário, cólon, rins e pulmão, [46, 51] contribuindo para a possível suscetibilidade destes tumores à ação citotóxica das células NK.

Observações clínicas implicaram as células NK no restabelecimento de pacientes submetidos ao transplante haploidêntico com células tronco-hematopoiéticas para o tratamento da leucemia mieloide. [52] Foi também observado que pacientes com neoplasias epiteliais com infiltrados linfocitários ricos em células citotóxicas, linfócitos T CD8 e NK, apresentam melhor prognóstico. [53, 54, 55] Tais observações combinadas com a melhor compreensão de como as interações receptor-ligante influenciam na ação citotóxica das células NK contra as células tumorais tem fomentado estudos abordando seu potencial uso terapêutico, tanto para o tratamento autólogo como alogênico do câncer. [42, 43, 48, 56, 57]

Neste sentido, o efeito anti-leucêmico das células NK foi observado tanto em pacientes tratados com células NK alogênicas purificadas, [58] quanto em camundongos imunodeficientes enxertados com células leucêmicas humanas e tratados com células NK expandidas *in vitro.* [15] Células NK de pacientes com mieloma múltiplo, expandidas *ex vivo,* apresentaram ação citotóxica contra células tumorais autólogas mantendo sua tolerância contra células autólogas normais. [28] Carlsten e colaboradores [19] observaram que células NK alogênicas de doadores saudáveis não estimuladas, foram capazes de reconhecer e lisar células tumorais primárias de ovário com baixa expressão do HLA de classe I,

indicando o carcinoma de ovário como alvo potencial para terapia baseada no transplante de células NK. Foi também observado que as células NK de pacientes com câncer de ovário apresentaram pouca ou nenhuma atividade antitumoral contra tumorais primárias de ovário, linhagens tumorais ovarianas, ou mesmo, contra as células K562, as quais são consideradas células alvo padrão em ensaios de citotoxicidade celular com células efetoras humanas. [59, 60] Todavia, a atividade citolítica contra células tumorais de ovário foi restaurada ao se estimular as células efetoras de pacientes com câncer de ovário com IL-2 ou por meio do enriquecimento da preparação de células efetoras com *large granular lymphocytes*, os quais correspondem às células NK. [61]

Outra célula capaz de lisar células tumorais são as NK-*like* T. As NK-*like* T constituem um subtipo de linfócito T que expressa, em sua superfície, vários receptores característicos das células NK (CD16, CD56, CD57, CD161, CD94/NKG2A) e, portanto, são identificadas pelo fenótipo $CD3^{+}CD56+$. [62, 63, 64] Todavia, é importante se distinguir entre as células NK-T invariantes e as NK-*like* T convencionais que também apresentam o fenótipo $CD3^{+}CD56^{+}$. A ativação das células NK-*like* T não está restrita ao reconhecimento por meio do receptor CD1d como ocorre nas células NK-T invariantes e, ainda, as NK-*like* T não expressam as cadeias invariantes do receptor TCR (Vα24JαQ e Vβ11). [65] Considera-se que a ação citolítica das células NK-*like* T possa ser mediado tanto pelo reconhecimento específico da molécula HLA I, característico dos linfócitos T citotóxicos, quanto pelo reconhecimento de ligantes inibidores e ativadores, característico das células NK. [64, 66] A ação antitumoral das NK-*like* T tem sido documentada contra uma variedade de células tumorais, incluindo células tumorais autólogas de ovário.[67] Em geral, as células NK-*like* T constituem uma pequena porcentagem dos linfócitos circulantes e o aumento no

número destas células tem sido associado à ativação crônica do sistema imune, como ocorre nas infecções por vírus latentes, neoplasias e doenças autoimunes. [65] As células NK-*like* T, assim como as células NK, podem ser expandidas a partir das CMSP por meio do cultivo celular de longa duração suplementado com citocinas como INF-γ e IL-2 combinados ao anti-CD3. [15, 67, 68, 69, 70]

Objetivos

2.OBJETIVOS

2.1 Objetivo geral

Avaliar a atividade funcional das células NK e NK-*like* T do sangue e ascite de pacientes com neoplasia de ovário.

2.2 Objetivos específicos

Determinar a expressão do marcador de degranulação CD107a nas células NK e NK-*like* T do sangue e ascite.

Comparar o efeito de dois procedimentos de cultivo celular, um de curta duração (18h) e outro de longa duração (21 dias) na expressão do CD107a e na expressão dos receptores ativadores DNAM-1, NKp30 e NKp44 das células NK e NK-*like* T do sangue periférico.

Relacionar a expressão dos receptores ativadores com degranulação das células NK e NK-*like* T.

Sujeitos e Métodos

3. SUJEITOS E MÉTODOS

3.1 Desenho do estudo

Trata-se de um estudo descritivo e prospectivo do comportamento biológico das células imunes humanas providas de citotoxicidade natural, principalmente das células NK, no sentido de descrever a resposta proliferativa e a atividade anti-tumoral quando estimuladas dentro de condições adequadas de cultura celular e co-incubadas com células tumorais de linhagem.

3.2 Sujeitos

Foram incluídas no estudo 24 pacientes, segundo critérios previamente estabelecidos e homologados pelo Comitê de Ética em Pesquisa (CEP 489/2005 e CEP 897/2011), respeitando os princípios enunciados na Declaração de Helsinque e na resolução CNS 196/96 do Ministério da Saúde. Assim, as pacientes aceitaram participar voluntariamente do estudo após receberem esclarecimentos quanto aos objetivos da pesquisa e procedimentos a que seriam submetidas. A anuência da paciente em participar da pesquisa deu-se pela assinatura do Termo de Consentimento Livre Esclarecido (Anexo 1). As características dos sujeitos no momento da inclusão no estudo e obtenção das amostras de sangue e/ou ascite estão na tabela 1. O estádio clínico das pacientes e o diagnóstico histológico dos tumores seguiram os critérios de classificação estabelecidos pela Federação Internacional de Ginecologia e Obstetrícia (FIGO) (Anexo 2). As pacientes foram agrupadas segundo as características dos tumores, se benigno ou maligno, e a presença de metástases, resultando nos seguintes grupos experimentais: alterações

anexiais benignas (Bng, n=10), neoplasia maligna sem mestástases (Mlg I-II, n=7) e neoplasia maligna com mestástases (MlgMt III-IV, n=7).

3.3 Critérios de inclusão

Foram incluídas neste estudo pacientes do “Hospital Prof. Dr. José Aristodemo Pinotti” - Centro de Atenção Integral a Saúde da Mulher (CAISM) com carcinoma epitelial de ovário (estágios I a IV) e tumores benignos de ovário classificados de acordo com a Federação Internacional de Ginecologia e Obstetrícia (FIGO).

3.4 Coleta de dados dos sujeitos

Informações sobre os sujeitos, relevantes para a realização do estudo, foram obtidas nos prontuários médicos das pacientes, mantidos pelo Serviço de Arquivo Médico (SAME) do Hospital da Mulher Prof Dr José Aristodemo Pinotti – CAISM. Estas informações foram armazenadas em um banco de dados Excel e incluem: codificação do sujeito, data de nascimento, valores dos marcadores CEA e CA125, resultado da ultrassonografia, hipótese diagnóstica, data e tipo de cirurgia realizada, diagnóstico patológico de congelação, diagnóstico patológico final, estadiamento clínico, presença de ascite, diagnóstico citológico da ascite, tratamento por quimioterapia. As características histopatológicas das pacientes podem ser visualizadas no anexo 3.

As amostras de sangue foram colhidas através de punção venosa utilizando o sistema de coleta a vácuo em tubos com capacidade para 9ml contendo heparina sódica (Vacuette, Campinas, Brasil). Todas as amostras de sangue foram obtidas por ocasião da internação das pacientes para cirurgia diagnóstica no CAISM.

3.5 Colheita e processamento das amostras de sangue e ascite

A colheita de sangue foi realizada pelo Serviço de Enfermagem logo após a internação da paciente para cirurgia diagnóstica ou citorredução do câncer de ovário. Foram colhidos 2 tubos com capacidade para 9 ml de sangue por meio de punção venosa utilizando sistema de colheita a vácuo com tubos contendo heparina sódica (Vacuette, Campinas, Brasil). As células mononucleares do sangue periférico (CMSP) foram isoladas por gradiente de centrifugação (Ficoll, Amersham Pharmacia Biotech, Psicataway, NJ, USA) e criopreservadas em meio SFB (Hyclone, Logan, USA) contento 10% de DMSO (Sigma, St Louis, USA) em nitrogênio líquido, até o momento do uso. Ascites, não carcinomatosas, foram obtidas de 6 pacientes durante o procedimento cirúrgico, até o volume de 100ml. A fração de células mononucleares presente nas ascites (CMA) foi isolada por gradiente de centrifugação (Ficoll, Amersham Pharmacia Biotech, Psicataway, NJ, USA), lavadas (centrifugação 400g/10min) duas vezes com PBS (LCG Biotecnologia Ltda, Cotia, Brasil) e criopreservadas com meio SFB contento 10% de DMSO em nitrogênio líquido, até o momento do uso.

3.6 Cultura de linhagem de células tumorais K562

A linhagem tumoral eritromieloblastoide humana K562 foi mantida em meio RPMI-1640 (LCG Biotecnologia Ltda, Cotia, Brasil) suplementado com 10% de SFB (Hyclone, Logan, USA), 2mM L-glutamina. As células K562 foram mantidas por meio de repiques e adição de meio cultivo celular novo a cada 48h-72h, de modo que a densidade celular no frasco de cultivo variasse entre $0,3x10^6$ cél./ml a $1,2x10^6$ cél./ml. A cada 8-10 repiques as células K562 foram substituídas por células do estoque de células criopreservado.

3.7 Cultivo de curta duração das CMSP e CMA (pré-estimulação e curta-estimulação com IL-2)

Frascos de criopreservação contendo CMSP ou CMA foram retirados do container de nitrogênio líquido, descongelados à temperatura ambiente e as células foram lavadas duas vezes com PBS (Centrifugação 400g/10min). As células foram contadas em câmera de Neubauer usando-se solução de acido acético (2% v/v em PBS) ou solução de azul tripan (1% w/v em PBS) para determinação do número e viabilidade das células. Depois de lavadas, os precipitados de CMSP ou CMA foram suspensas com volume suficiente de RPMI-1640 suplementado com SFB (10%) e L-Glutamina (2mM) para obter-se densidade igual a $1x10^6$ cél./ml. A suspensão celular foi dividida em duas partes iguais e, a uma delas, foi adicionado 1000U/ml de rhIL-2 (BD Pharmigen, San Diego, EUA). Ambas as duplicatas foram incubadas por uma noite (16h) à 37^oC e 5% de CO_2. Por meio deste procedimento obtiveram-se para cada uma das amostras de CMSP e CMA as suspensões de células efetoras estimuladas por curta duração (curta-estimulação) com IL-2 e as células efetoras não estimuladas (pré-estimulação) posteriormente utilizadas no ensaio para a avaliação da atividade funcional dos linfócitos NK e NK-*like* T.

3.8 Cultivo de longa duração das CMSP (longa-estimulação com IL-2)

Para o cultivo de longa duração das CMSP utilizou-se o método descrito por Carlens e colaboradores [6], de expansão seletiva de células NK. Assim, 2ml da suspensão de CMSP, densidade igual a $0,5x10^6$ cél./ml, em meio CellGro SCGM (CellGenix, Freiburg, Alemanha) suplementado com 10% de SFB (Hyclone, Logan, USA), 10ng/ml de anti-CD3 (clone HIT3a, BD Pharmigen, San Diego, EUA) e 1000U/ml de rhIL-2 (BD Pharmigen, San Diego, EUA) foram lançadas em placas de petri (35x10mm, Corning, New York, NY, EUA) e incubadas (37^oC, 5% CO_2). No dia 6 do cultivo, as células foram raspadas, suspensas

com auxílio de uma pipeta e transferidas para um tubo de 15 ml. Foi determinado o volume total, numero de células e viabilidade pelo método de exclusão do azul-tripan. A suspensão celular foi centrifugada para remoção do meio contendo o anti-CD3. O precipitado celular foi suspenso com volume suficiente do meio CellGro SCGM suplementado com SFB (10%) e rhIL-2 (1000U/ml) para obter-se densidade final igual a $1x10^6$cél./ml e, finalmente, a suspensão celular foi transferido para frasco de cultura T-25 (TPP, Transadingen, Suíça) e incubadas (37^oC, 5% CO_2). Nos dias 10 e 15 do cultivo celular, o procedimento de manutenção realizado no dia 6 foi repetido, resultando na expansão da cultura. Além dos procedimentos de manutenção da cultura celular descritos para os dias 6, 10 e 15, foi adicionado rhIL-2 (1000U/ml) aos fracos de cultivo nos dias 3, 7, 8, 9, 13, 14, 16, 17 e 20. Por meio deste procedimento obtiveram-se as suspensões de CMSP efetoras estimuladas por cultivo celular de longa duração (longa-estimulação) com IL-2 posteriormente utilizadas no ensaio para a avaliação da atividade funcional dos linfócitos NK e NK-*like* T.

3.9 Atividade funcional dos linfócitos NK e NK-like T (ensaio de degranulação)

A avaliação da ativação funcional das células NK e NK-*like* T, bem como, a fenotipagem dos subtipos de linfócitos foram realizadas simultaneamente por meio da citometria de fluxo, empregando-se o método descrito por Bryceson e colaboradores. [71] A avaliação funcional das células NK e NK-*like* T baseou-se na expressão da molécula CD107a como marcador do processo de degranulação celular, indicando, dessa forma, a capacidade das células efetoras serem ativadas, quando coincubadas com células alvo (linhagem K562). [72]

As suspensões de células efetoras (E) obtidas pelo cultivo celular de curta duração (CMSP e CMA, pré-estimulação e curta-estimulação com IL-2) e cultivo celular de longa duração (CMSP longa-estimulação com IL-2) foram centrifugadas (400g/10min) e suspensas com volume suficiente do meio RPMI-1640 suplementado com SFB (10%) e L-Glutamina (2mM) para obter-se densidade igual a $2x10^6$ cél./ml. As células alvo (T) também foram preparadas centrifugando-se células K562 mantidas em cultura, seguindo-se a suspensão do precipitado celular com volume necessário de RPMI-1640 suplementado com SFB (10%) e L-glutamina (2mM) para obter-se densidade de células K562 viáveis igual a $2x10^6$ cél./ml.

Os ensaios de degranulação foram realizados coincubando-se células efetoras e alvo na proporção 1:1 (E:T), volume final igual a 200µl e em duplicata. Assim, 100ul da suspensão de células alvo foram pipetados em microtubos (capacidade para 0,6ml) com fundo cônico e tampa. A seguir, foram pipetados 100ul da suspensão de células efetoras, que poderiam ser CMSP pré-estimulação, CMSP curta-estimulação, CMSP longa-estimulação, CMA pré-estimulação e CMA curta-estimulação. A tabela 2 exemplifica um protocolo experimental, mostrando como as células efetoras pré-estimulação e curta-estimulação, bem como, as células alvo foram distribuídas nos microtubos de um mesmo ensaio. Os microtubos contendo as misturas de células efetoras:alvo (E:T) foram centrifugados a 30g/3min. e incubados por 2h/37^oC. Tubos contendo as células efetoras sem células alvo também foram preparados para quantificação basal da expressão do marcador CD107a. Depois da incubação, os microtubos foram centrifugados (600g/5min), os sobrenadantes foram descartados. Os precipitados celulares foram suspensos com 50ul da solução de marcação, a qual continha a mistura de anticorpos monoclonais

conjugados a fluoróforos e foi preparada da seguinte forma: Os anticorpos monoclonais anti-CD3 FITC (clone HIT3a, Becton Dickinson, Mountain View, CA, EUA), anti-CD56 PE (clone MEM188, Becton Dickinson, Mountain View, EUA) e anti-CD107a PECy5 (clone eBioH4A3, ebioscience, San Diego, EUA) foram diluídos na proporção 1:50 com a solução PBS suplementada com FBS (2%) e EDTA (2mM). As misturas celulares foram incubados na solução de marcação por 30minutos em banho de gelo e no escuro. Depois do período de incubação as células foram lavadas duas vezes por centrifugação (600g/3min), o precipitado celular final foi suspenso com 300ul da solução PBS suplementada com FBS (2%) e EDTA (2mM) e transferidas para tubos de citometria. As células, assim obtidas, foram analisadas por meio da citometria de fluxo, ajustando-se apropriadamente os canais SSC/FSC, de modo a localizar a população de células efetoras (linfócitos) para a aquisição de 10.000 - 20.000 eventos (Figura 1). Foi utilizado o programa FlowJo Software (Tree Star, Ashland, EUA) para a análise de dados.

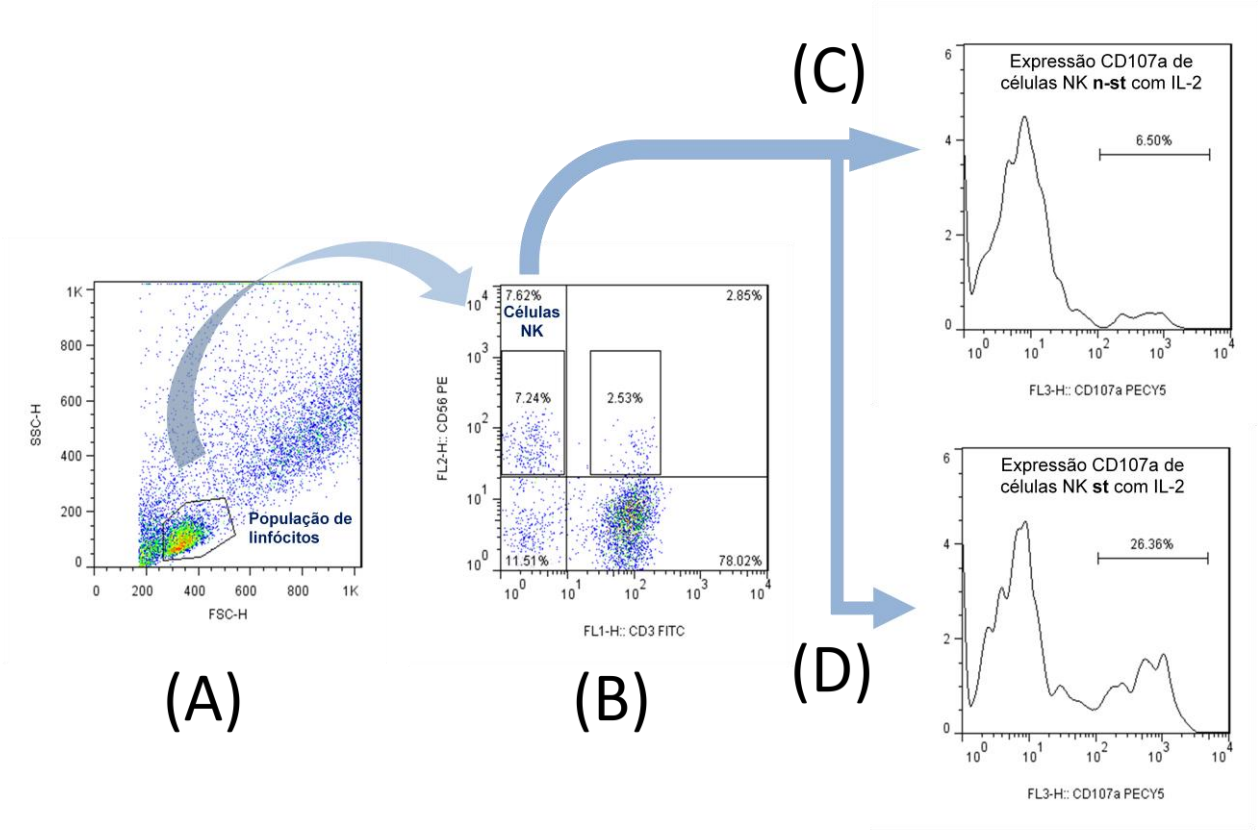

Figura 1: Método de análise usado na citometria de fluxo para avaliar a expressão do marcador celular CD107a nas células NK. (A) Morfologia das CMSP (FSC=tamanho celular, SSC=complexidade celular) e *gate* selecionando a população de linfócitos. (B) Subtipos de linfócitos determinados pela expressão dos marcadores CD3 e CD56 e *gate* selecionando a população de células NK. (C) Histograma mostrando a expressão do CD107a nas células NK não estimuladas (n-st) e (D) estimuladas (st) com IL-2.

3.10 Fenotipagem dos linfócitos e receptores ativadores DNAM-1, NKp30 e NKp44

Os subtipos de linfócitos, bem como a expressão dos receptores ativadores DNAM-1, NKp30 e NKp44 foi realizada por meio da citometria de fluxo, utilizando-se protocolo previamente padronizado combinando-se 4 cores simultaneamente. As células foram misturadas com quantidades apropriadas dos MAb conjugados a fluoróforos anti-CD3 APC-Cy7 (clone HIT3a), anti-CD56 PE-Cy5 (clone MEM188), anti-DNAM-1 FITC (clone DX11) (Becton Dickinson, Mountain View, EUA), anti-NKp30 PE (clone AF29-4D12) e anti-NKp44 PE (clone 2.29) (Miltenyi Biotec, Bergisch Gladbach, Alemanha). As células foram

incubadas por 30 minutos em banho de gelo e protegidas de luz. Depois da incubação, as células foram lavadas duas vezes (centrifugação 600g/5min) com PBS e os precipitados finais foram suspensos para aquisição, usando-se FACS Verse com programa FACS Suite. As células foram adquiridas após ajuste apropriado dos canais SSC/FSC, de modo a localizar a população de linfócitos para a aquisição de 10.000 eventos. Foi utilizado o programa FlowJo Software para a análise de dados.

3.11 Criopreservação

As suspensões de células (leucócitos, células efetoras ou linhagens neoplásicas) foram centrifugadas (600g/ 10min), tendo o sobrenadante desprezado e o precipitado suspenso com 1ml de SFB (LGC Biotecnologia, Brasil) contendo 10% de Dimethil sulfóxido (DMSO – SIGMA, St. Louis, USA). As suspensões foram transferidas para frascos próprios para congelação e armazenadas à -80^{o}C pelo período de 24-72 horas. Após este período, os frascos foram transferidos para nitrogênio líquido.

3.12 Análise de resultados e estatística

A expansão celular (número de vezes=número de células no momento/número de células no dia 0) foi calculada baseando-se no número de células viáveis. As comparações de variáveis entre grupos foram realizadas pelo teste-t de Student ou teste de Mann-Whitney e para amostras dependentes dentro dos grupos foi utilizado o teste-t de Student. Foi realizado a regressão linear Stepwise para avaliar o efeito das variáveis (DNAM-1, NKp30 e NKp44) na ativação das células efetoras. O nível de significância foi estabelecido em $p < 0,05$.

Publicação

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4. PUBLICAÇÃO

Up-regulation of DNAM-1 and NKp30, associated with improvement of NK cells activation after long-term culture of mononuclear cells from patients with ovarian neoplasia.

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Abstract

NK cells are lymphocytes known by their ability to eliminate a variety of malignant cells in a process involving innate recognition. Similarly, NK-like T lymphocytes have been reported to eliminate tumor cells, but the targeting process might involve either innate or adaptive immune recognition. This study aimed at evaluating the activation of NK and NK-like T cells from blood and ascites of ovarian neoplasia patients. The effects of two culture procedures (short and long-term) on degranulation and expression of activating receptors of NK and NK-like T cells from blood were also compared. Blood from patients with adnexial benign alterations (n=10) and ovarian neoplasia (grade I-II n=7 and grade III-IV n=7) were collected after signed consent. Ascites without neoplasia cells were obtained from 6 of the patients. Mononuclear cells from blood and ascites were isolated by gradient centrifugation. The functional activation of NK and NK-like T cells were evaluated by the expression of CD107a molecule, while coincubated (ratio 1:1) with K562 target cells. Short-term culture was conducted overnight in RPMI-1640 medium supplemented with IL-2 (1000UI/ml), FBS (10%) and L-glutamine (2mM). Long-term culture was conducted by a 21 day process with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), IL-2 (1000UI/ml) and FBS (10%). NK activation seemed to be impaired as the disease worsens. Both culture procedures increased NK cells activation significantly, but long-term was much higher than short-term ($p<0.05$). NK-like T lymphocytes showed low activation, which was not altered by the culture procedures. DNAM-1 expression was decreased on NK and NK-like T cells of patients with malignant neoplasia. However, long-term culture increased significantly ($p<0.05$) the expression of DNAM-1 on NK-like T and also the expression of NKp30 on NK lymphocytes. Stepwise linear regression evaluating

the effect of variables DNAM-1, NKp30, NKp44 on effector cells activation, indicated that NKp30 was the most effective receptor on NK cell activation ($p < 0.0003$) and DNAM-1 on NK-like T cell activation ($p < 0.0248$). As a conclusion, the long-term culture system employed resulted in a high number of functional NK cells. This method was particularly efficient to up-regulate NKp30 and DNAM-1 on NK cells. The poor degranulation observed in NK-like T lymphocytes suggests that, in the experimental system employed, these cells might not be directly involved through the innate pathway targeting process.

Introduction

Ovarian carcinoma is the most lethal gynecological cancer, being among the main causes of women's death. Its lethality is a consequence of the advanced stage of the disease at the moment of diagnosis. [1, 2] The fast metastatic dissemination to the peritoneal cavity and to lymph nodes hinders the efficiency of regular therapies. Therefore, new therapeutic strategies based on the immune system manipulation have been proposed for elimination of metastatic cancer cells and prevention of relapse. In this context, both NK and NK-like T cells have demonstrated to play a role against ovarian carcinoma cells. [3, 4, 5, 6]

Natural killer (NK) cells constitute 5–15% of the lymphocytes in human peripheral blood, being recognized by the expression of CD56 and absence of CD3 surface molecules. These cells were primarily identified by their capacity of eliminating a variety of tumor cells without previous stimulation. [7, 8] Their cytotoxic capacity is due to a process involving innate recognition of the target cells by an array of stimulatory and inhibitory receptors. Inhibitory ligand stimuli are recognized by HLA class I-binding receptors, such as killer cells Ig-like receptors (KIRs), CD94/NKG2A and leukocyte Ig-like receptor B1 (LILR-B1). Activating ligand stimuli are recognized by a variety of activating receptors, highlighting DNAX accessory molecule-1 (DNAM-1), natural cytotoxicity receptors (NKp30, NKp44, NKp46) and NKG2D, as the most important ones. [9, 10, 11, 12, 13] Therefore, the regulation of NK cells cytotoxic function is established by a balance between the activating and inhibitory receptors upon interaction with their ligands on target cells. Additionally, different combinations of NK cell activating receptors drive distinct responses through synergistic effects, thus affecting their activation function. [14, 15]

The NK-like T cells are another lymphocyte subset which has also been reported to eliminate tumor cells, but the targeting process might involve either innate or adaptive immune recognition. [16, 17, 18] These cells are characterized by the expression of some of the surface markers found on T lymphocytes and on NK cells simultaneously. Although they represent a low percentage of circulating lymphocytes, NK-like T cells have been reported to expand in long-term peripheral blood mononuclear cells (PBMC) cultures supplemented with cytokines such as INF-γ and IL-2 together with anti-CD3. [3, 6, 18, 19] The NK-like T cells expanded under these culture conditions are distinct from invariant NKT cells because they are not restricted to CD1d and do not express the invariant T cell receptor. [20]

Similarly to other cancers, ovarian cancer exploits an array of immunological ways to create a suppressive environment to prevent being eliminated by the immune response. [21, 22] NK cells from patients with ovarian carcinoma have been reported to display none or poor cytotoxic function against ovarian cell line, fresh ovarian tumours and even against the prototype NK cell target K562. [21, 23, 24, 25, 26,] NK antitumour activity has been reported to be significantly lower among patients with ovarian carcinoma than in patients with benign masses. [27] Particularly, the DNAM-1/CD155 receptor-ligand interaction plays an important role on the NK targeting mechanism against ovarian carcinoma cells in humans. [4] However, it has been shown down-regulation of DNAM-1 expression on NK cells from patients with ovarian carcinoma as a consequence of repetitive exposure to CD155, a molecule that often is over expressed on ovarian carcinoma cells. [28] In spite of the functionality of NK cells being impaired in cancer patients, ex vivo culture procedures or adoptive transfer of NK and NK-like T cells have been shown to provide functional effector cells that could be used for immunotherapy of cancer. Therefore, this study aimed at

evaluating the functional activation of NK and NK-like T cells from blood and ascites of ovarian neoplasia patients. The effects of two culture procedures employing IL-2, one short-term (18h) and another long-term (21 days) designed to expand the CD56+ lymphocytes, was also compared in relation to the activation and the expression of activating receptors DNAM-1, NKp30 NKp44 on NK and NK-like T cells from blood of patients with ovarian neoplasia.

Material and methods

Patients, blood and ascite samples

In this study, 24 patients with adnexial masses and scheduled for surgical intervention were included from 'Hospital da Mulher – Prof Dr José Aristodemo Pinotti – Centro de Atenção Integral à Saúde Mulher', the women's hospital of Campinas University (Unicamp), Campinas, Brazil. The study was approved by the Research Ethics Committee of Unicamp, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Patient characteristics at the time of blood sampling are provided in Table 1. Blood samples were collected by using 9 ml vacuum blood-sampling tubes containing sodium heparin (Vacuette, Campinas, Brazil). Ascites samples were obtained from six patients with malignant neoplasia, without carcinomatosis, during the surgical intervention. Ascitis were harvested from peritoneal cavity, transferred to 50ml tubes and, immediately added with sodium heparin (5000U/ml, Liquemine Roche, Rio de Janeiro, Brazil). Peripheral blood mononuclear cells (PBMC) and ascitis mononuclear cells (AMC) were isolated by gradient centrifugation, using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden). PBMC and AMC were washed twice with balanced salt solution (PBS; LCG Biotecnologia Ltda, Cotia, Brazil) and cell number and viability were assessed by trypan

blue exclusion. Replicates of the resulting cell pellet was frozen in foetal bovine serum (FBS; Hyclone, SFM4Transfx-293, Logan, Utah, USA) containing 10% DMSO (Sigma, St Louis, USA) for subsequent phenotyping and activation experiments.

Non and Short-term rhIL-2 stimulated PBMC and AMC

Cryovials with PBMC or AMC were taken out of the liquid nitrogen container, thawed at room temperature and washed twice with PBS. The cells were counted in a Neubauer chamber using acetic acid solution (2% v/v in PBS) and trypan blue solution (1% w/v in PBS) to assess viability. After washing, the final PBMC or AMC pellet was suspended at 1x106 cells/ml with RPMI-1640 supplemented with FBS (10%) and L-Glutamine (2mM). The cell suspension was split in two equal parts and, to one of the replicates, 1000U/ml of rhIL-2 was added. Both replicates were incubated over-night at 37oC and 5%CO2.

Long-term culture PBMC

The culture method described by Carlens et al. [29] was used as a base for the long-term culture, which was able to generate effector cell suspensions enriched with NK and NK-like T cells. PBMC from eight patients with adnexial benign alterations and four patients with malignant ovarian neoplasia (one grade I and three grade III) were used in this procedure. Two ml of PBMC suspension at a density of 0.5x106 cells/ml in CellGro SCGM (CellGenix, Freiburg, Germany) medium containing 10% of FBS, 10ng/ml of anti-CD3 (clone HIT3a, BD Pharmigen, San Diego, USA) and 1000U/ml of rhIL-2 (BD Pharmigen, San Diego, USA) were plated in petri dishes (35 X 10 mm; Corning, New York, NY, USA) and taken to an incubator (37oC, 5% CO2). On day 6 the cells were scraped, suspended thoroughly with a pipette and transferred to a 15ml tube. Total volume, number of cells and

viability were determined. The cell suspension was centrifuged to washed-out the medium containing anti-CD3. The cell pellet was suspended in fresh CellGro SCGM medium, supplemented with FBS (10%) and rhIL-2 (1000U/ml) at a density of 1x106/ml, then transferred to a T-25 cell culture flask (TPP, Transadingen, Switzerland) and taken to an incubator (37oC, 5% CO2). On days 10 and 15, the same procedure as day 6 was conducted in order to keep the cells expanding until day 21. Additionally to the cell culture maintenance, on days 6, 10 and 15, rhIL-2 (1000U/ml) was further provided to the culture on days 3, 7, 8, 9, 13,14,16,17 and 20.

K562 cell line culture

The K562 (human erythromyeloblastoid) cell line originally obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) is regularly maintained in the laboratory. The K562 cells were grown in vitro in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine. The cultures were replenished with fresh medium every 2–3 days throughout the culture period. Cell line cultures were replaced by new cells from the frozen stock after 8–10 in vitro passages and routinely phenotyped for the main surface markers characterizing this cell line (data not shown).

Lymphocytes phenotyping

Non and short-term rhIL-2 stimulated PBMC and AMC, and long-term (on days 0, 10 and 21) cultivated PBMC were phenotyped for identification of the lymphocyte subtypes and expression of activating receptors on the CD56+ cells. A flow citometric-based assay combining four colours was used according to standards procedures. The cells were mixed with appropriate concentrations of fluorochrome-conjugated monoclonal antibodies (MAb)

anti-CD3 APC-Cy7 (clone HIT3a), anti-CD56 PE-Cy5 (clone MEM188) and anti-DNAM-1 FITC (clone DX11) (Becton Dickinson, Mountain View, USA), and anti-NKp30 PE (clone AF29-4D12) and anti-NKp44 PE (clone 2.29) (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were incubated for 30 minutes in ice bath and protected from light. After the incubation, cells were washed twice (centrifuged at 400g / 5min) with PBS and the final cell pellets suspended for acquisition, using a FACS Verse with FACS Suite software (Becton Dickinson, San Jose, USA). All samples were analyzed by setting appropriate SSC / FSC gates on the lymphocyte population. FlowJo software (Tree Star, Ashland, USA) was used for the data analysis.

Functional activation of NK and NK-like T lymphocytes

A flow cytometric assay, based on the expression of the CD107a molecule (LAMP-1) on the cell surface, was employed for the evaluation of NK and NK-like T lymphocytes functional activation. The method was based on the former described by Bryceson et al [30] for the evaluation of NK functional activation during target cell lysis. Effector cells (non and short-term IL-2 stimulated PBMC / AMC, and long-term cultivated PBMC) and target cell (K562) suspensions were prepared at 2x106 cells/ml. Each of the effector cell suspensions were coincubated in duplicate with target cells at a 1:1 ratio in U bottom microtubes, with a final volume of 200µl. Cells were spun-down quickly (30g / 3min) and taken to an incubator during 2h at 37oC. Tubes containing effector cells without target cells were also prepared for quantification of basal expression of CD107a. After incubation, microtubes were centrifuged (600g / 5min), supernatants were discarded and cell pellets were suspended with 50ul of cold stain solution (PBS supplemented with 2% FBS and 2mM ethylenedianminetetraacetic acid) containing the fluorochrome-conjugated monoclonal

antibodies (MAb) anti-CD3 FITC (clone HIT3a, Becton Dickinson, Mountain View, CA, USA), anti-CD56 PE (clone MEM188, Becton Dickinson, Mountain View, USA) and anti-CD107a PECy5 (clone eBioH4A3, ebioscince, San Diego, USA). Cells were incubated for 30 minutes in ice bath and protected from light. After the incubation, cells were washed twice (centrifuged at 400g / 5min) and the final cell pellets suspended with 300ul of staining solution. Data acquisition was performed using a FACS Verse with FACS Suite software. A range of 10,000 to 20,000 cells were acquired. All samples were analyzed by setting appropriate SSC / FSC gates on the lymphocyte population. FlowJo software was used for the data analysis.

Statistics and calculations

Cell expansion (fold = cell no. at a given time-point／cell no. at day 0) was calculated based on viable cells. Comparisons of variables between groups were performed by Student's t-test or Mann–Whitney test and within groups by Student's t-test for dependent samples. Stepwise linear regression was performed to evaluate the effect of variables (DNAM-1, NKp30 and NKp44) on effector cell activation. The level of significance was set at p-value < 0.05.

Results

Patients and groups

In this study, 24 patients with adnexial masses were included. Patients were grouped according to the ovarian alteration characteristics (benign or malignant), presence of metastasis and origin of the sample (blood or ascites), resulting in the following groups: adnexial benign alterations (Bgn, n=10), malignant grade I-II (I-II, n=7), malignant grade III-

IV (III-IV, n=7) and ascites (Asc, n=6). Table 1 shows the patient characteristics at the time of blood sampling.

Functional activation of NK and NK-like T lymphocytes

NK functional activation seemed to be impaired with the progression of the disease, as assessed by the percentage of NK cells expressing CD107a in non rhIL-2 stimulated PBMC coincubated with the target K562 cells (median%/variation, Bng=12.25/ 2.25-21.78; grade I-II=9.66/ 6.48-15.48; grade III-IV=7.73/ 3.65-10.06). The exposure of the effector cells to the tumor site also seemed to affect NK cell function, since non rhIL-2 stimulated AMC showed the lowest percentage of NK cells expressing CD107a molecule after coincubation with target cells (Asc=6.99/ 1.68-21.68) (Figure 1A). Short-term cultures of PBMC and AMC with rhIL-2 (short-term culture, figure 1B) increased significantly the percentage of NK cells expressing CD107a molecule after coincubation with target cells (median%/ variation: Bng=26.19/ 9.52-57.03; grade I-II=17.81/ 8.69-52.37; grade III-IV=17.59/ 9.28-35.63; Asc=33.55/ 3.83-43.50). The long-term culture system (long-term culture, figure 1C), employed to expand CD56+ effector cells from PBMC of patients with benign adnexial alterations (n=8) and malignant ovarian neoplasia (n=4), increased twice as much the percentage of NK cells expressing CD107a molecule after coincubation with target cells, than the short-term IL-2 stimulated cultures (median%/ variation: Bng=58.04/ 40.39-74.48; grade I-IV=48.33/ 33.89-54.76). The long-term culture increased not only the number of NK cells activated, but also the degranulation magnitude, as assessed by the CD107a fluorescence intensity (Figure 1C, histograms).

NK-like T lymphocytes showed low activation when non rhIL-2 stimulated PBMC and AMC were coincubated with K562 target cells, as assessed by the percentages of NK-like

T lymphocytes expressing CD107a (Figure 2A; median%/variation: Bgn=3.92/ 1.67-11.23; grade I-II=6.25/ 1.70-13.34; grade III-IV=3.66/ 0.98-11.11; Asc=9.90/ 2.52-34.92). The short-term culture of PBMC and AMC with IL-2 (sort-term IL-2 stimiulated) didn't alter significantly the percentages of NK-like T lymphocytes expressing CD107a in relation to the pre IL-2 stimulated PBMC (Figure 2B; median%/ variation: Bgn=5.36/ 1.52-14.74; grade I-II=7.19/ 0.0-16.97; grade III-IV=4.55/ 2.53-18.70; Asc=12.39/ 4.48-35.38). NK-like T lymphocytes present in the long-term culture cell preparations seemed unresponsive to the coincubation with target cells, as assessed by the lowest percentages of cells expressing CD107a molecule observed (Figure 2C; median%/ variation: Bgn=2.57/ 1.10-11.06; grade I-IV=4.33/ 1.82-6.76).

Activating receptors

The expression of DNAM-1 seemed to be impaired in patients with malignant ovarian neoplasia since, in these patients, the percentage of NK and NK-like T lymphocytes expressing DNAM-1 in non and short-term IL-2 stimulated PBMC was lower than in patients with benign alterations (Figure 3A and B, DNAM-1). However, long-term culture of PBMC up-regulated the expression of DNAM-1 on CD56+ lymphocytes and resulted in significant increase ($p<0.05$) in the percentages of NK-like T cells expressing this receptor. The long-term culture also increased significantly ($p<0.05$) the percentage of NK cells expressing NKp30 in both patient groups (medians in Benign: non IL-2 stimulated=14.83%, short-term IL-2 stimulated=20.84%, long-term cultured=89.14% and medians in Malignant: non IL-2 stimulated=5.78%, short-term IL-2 stimulated=13.44%, long-term cultured=89.17%) (Figure 3A, NKp30). The percentage of NK cells expressing NKp44 was significant increased after long-term culture of PBMC (Figure 3A, NKp44).

However, NKp30 and NKp44 were not only poorly expressed by NK-like T lymphocytes, but also remained unchanged after the culture procedures (Figure 3B, NKp30 and NKp44). Stepwise linear regression evaluating the effect of variables DNAM-1, NKp30, NKp44 on effector cells activation, indicated that NKp30 was the most effective receptor on NK cell activation ($p< 0.0003$) and DNAM-1 on NK-like T cell activation ($p<0.0248$).

Selective expansion of CD56+ effector cells

Figure 4 shows the absolute numbers of lymphocyte subtypes (NK, NK-like T and T) on day 0 and day 21 of the long-term cultures from PBMC of the patients with adnexial benign alterations (n=8) and malignant ovarian neoplasia (n=4). At the start of the cultures (day 0), PBMC from patients with malignant ovarian neoplasia and benign alterations comprised similar amounts of lymphocyte subtypes. As expected, PBMC from patients with benign alterations cultured for 21 days resulted in CD56+ (NK and NK-like T) enriched cell preparations. At the end of the 21 days period of culture, PBMC from patients with adnexial benign alterations had expanded on average 463.9-fold (range: 40.4- to 928.4-fold); of these, NK cells had expanded on average 1058.8-fold (range: 26.0- to 2362.0-fold), NK-like T cells 2173.0-fold (range: 286.9- to 4372.2-fold) and T cells 352.4-fold (range: 26.8- to 919.1-fold). In patients with malignant ovarian neoplasia, PBMC cultured for 21 days had expanded on average 447.6-fold (range: 190.5- to 679.4-fold). Among these, NK cells had expanded on average 1175.1-fold (range: 241.6- to 2787.5-fold), NK-like T cells 1655.5-fold (range: 643.9- to 2791.2-fold) and T cells 495.6-fold (range: 146.9- to 902.1-fold). Nevertheless, as a consequence of the unequal expansion within the lymphocyte subtypes (NK, NK-like T and T), day-21 cultured cells of the benign alterations group presented a relative increase (% day-21 minus % day- 0) in NK (+9,6 ± 16.3%) and NK-like T cells

(+17.2 ± 16.7%) associated with a decrease in T lymphocytes (-16.1 ± 19.2%), while in the malignant group, there was a relative increase in NK (+7.6 ± 19.9%) and NK-like T cells (+7.4 ± 2.7%) associated with a poor relative decrease in T lymphocyte (-1.1 ± 19.1%).

Discussion

In this study NK and NK-like T cells from blood and ascites of ovarian neoplasia patients were successfully evaluated in relation to functional activation and expression of the activating receptors DNAM-1, NKp30 and NKp44. Furthermore, two culture procedures employing rhIL-2 were performed with PBMC from patients and compared in terms of stimulation and expression of activating receptors on NK and NK-like T lymphocytes.

Most of the existing knowledge on the functional integrity of the NK cells from patients with ovarian cancer was obtained by the traditional cellular cytotoxicity assay, which informs the percentage of dead target cells. Few reports, such as those by Carlsten et al [4, 28] have approached the functional integrity of the NK cells from ovarian cancer patients by directly observing the effector cells activation. Similarly, the functional activity of NK-like T lymphocytes from ovarian cancer patients has been only assessed by the traditional cellular cytotoxicity assay and, to our knowledge, exclusively with long-term cultivated effector cells [3, 5]. In the present study, the functional activity was evaluated by directly observing NK and NK-like T lymphocytes, which was achieved by assessing the CD107a molecule on the effector cell surface. The detection of CD107a molecule renders the evaluation of the functional activation through granule exocytosis of NK and NK-like T cells. [30,18]

Ours results showed that less than 25% of NK and NK-like T (Figures.1A and 2A) lymphocytes from freshly isolated PBMC or AMC of patients with benign and malignant ovarian neoplasia were responsive to the coincubation with the K562 target cells, as assessed by the CD107a expression. Additionally, NK cells were also responsive to the short-term rhIL-2 stimulation (Figure.1B), showing an increase in the percentage of cells expressing the CD107a molecule, while the percentage of NK-like T lymphocytes expressing CD107a remained unchanged (Figure.2B). The long-term culture procedure used in this study was based on the method described by Carlens et al. [29] This method employs CellGro SCGM medium supplemented with anti-CD3 and IL-2 and was originally described for selective expansion of NK cells from PBMC of healthy donors. In our study, this method supported not only the NK, but also the NK-like T cell expansion. PBMC cultures from patients with benign adnexial alteration progressed as expected, having as a result a relative increase in the CD56+ subtypes (NK and NK-like T lymphocytes) together with a relative decrease in the CD3+CD56- cells (T lymphocytes). However, the PBMC cultures from patients with malignant ovarian neoplasia progressed differently, because T lymphocytes had a poor relative decrease and, as a consequence, resulted in a higher absolute number of T lymphocytes at the end of the culture period, when compared to the PBMC cultures from patients with benign alterations (Figure.4).

Similar results were found by Alves et al, [6] who also demonstrated an association between the lymphocyte subtypes profile and altered cytokine production, after using similar culture procedure. They found that PBMC culture cytokine production from patients with malignant ovarian neoplasia was characterized by significantly lower INF-γ, TNF-α and higher IL-4, compared to patients with benign ovarian neoplasia. [6] Thus, both studies

indicate that ovarian malignant neoplasia affects the culture outcome. In spite of that, the long-term culture procedure from PBMC of patients with both benign and malignant alterations, was able to produce highly functional NK cells, as assessed by the CD107a expression (Figure.1C). Nevertheless, the culture procedure employed in this study was not as efficient as procedures reported in previous studies, [3, 18] which promoted functional activation in NK-like T lymphocytes. In this study NK-like T lymphocytes were poorly functional against K562 target cells (Figure. 2C). A possible explanation for that might involve the low expression of NKp30 observed on NK-like T lymphocytes. This argument is supported by a previous study using antibody blocking assay, which demonstrated that NK-like T cytotoxic function is dependent on NKp30 activating receptor. [18]

Although the stepwise linear regression has indicated NKp30 as the most effective receptor on NK cells activation, it has also been reported that DNAM-1 and NKp44 play a role on effector cells activation. [4, 28, 31] The long-term culture procedure employed in this study up-regulated all of these receptors on NK cells. Additionally, the possibility of generating DNAM-1-expressing NK cells would be particularly beneficial to overcome down-regulation often seen on patients with ovarian carcinoma and entitling this method for adoptive therapy. As a conclusion, the long-term culture system employed for the PBMC culture from ovarian neoplasia patients resulted in a high number of functional NK cells. Particularly, the culture system was efficient to up-regulate NKp30 and DNAM-1 receptors on NK cells. The poor degranulation observed in NK-like T lymphocytes suggests that, in the experimental system employed, these cells might not be directly involved through the innate pathway targeting process.

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Table 1. Patient characteristics (adnexial benign alterations n=10, malignant ovarian neoplasia n=14) at the time of blood sampling. Staging classifications followed FIGO Committee on Gynecologic Oncology guidelines.

Patient Code	Age (years)	Stage (FIGO)	Histological classification
19	68		Follicular cists
20	48		Fibroma
21	53	IIB	Serous adenocarcinoma
22	45		Mucinous cistadenoma
23	45	IIIC	Serous papillary adenocarcinoma
24	60	IIIC	Serous adenocarcinoma
27	65	IIIC	Serous adenocarcinoma
28	71		Serous cistadenoma
29	43		Follicular cists
30	59	IIIC	Serous adenocarcinoma
31	79	IIIC	Mucinous Adenocarcinoma
32	43		Serous papillary cistadenoma
33	62		Teratoma STRUMA OVARII
34	81	IC	Serous papillary adenocarcinoma
35	48	IA	Endometrioid adenocarcinoma
38	47		Mucinous cistadenoma
39	79		Mucinous cistadenoma
40	47	IIIC	Serous adenocarcinoma
41	54	IIA	Serous adenocarcinoma papillary
42	45		nd
43	53	IA	Sero-mucinous papillary boderline
44	25	IA	Serous papillary borderline
45	76	IV	Mucinous adenocarcinoma
48	77	IA	Serous carcinoma

*All histopathological reports were issued from Division of Pathology of the Clinical Hospital at University of Campinas. nd=non-determined since patient didn't go through surgery.

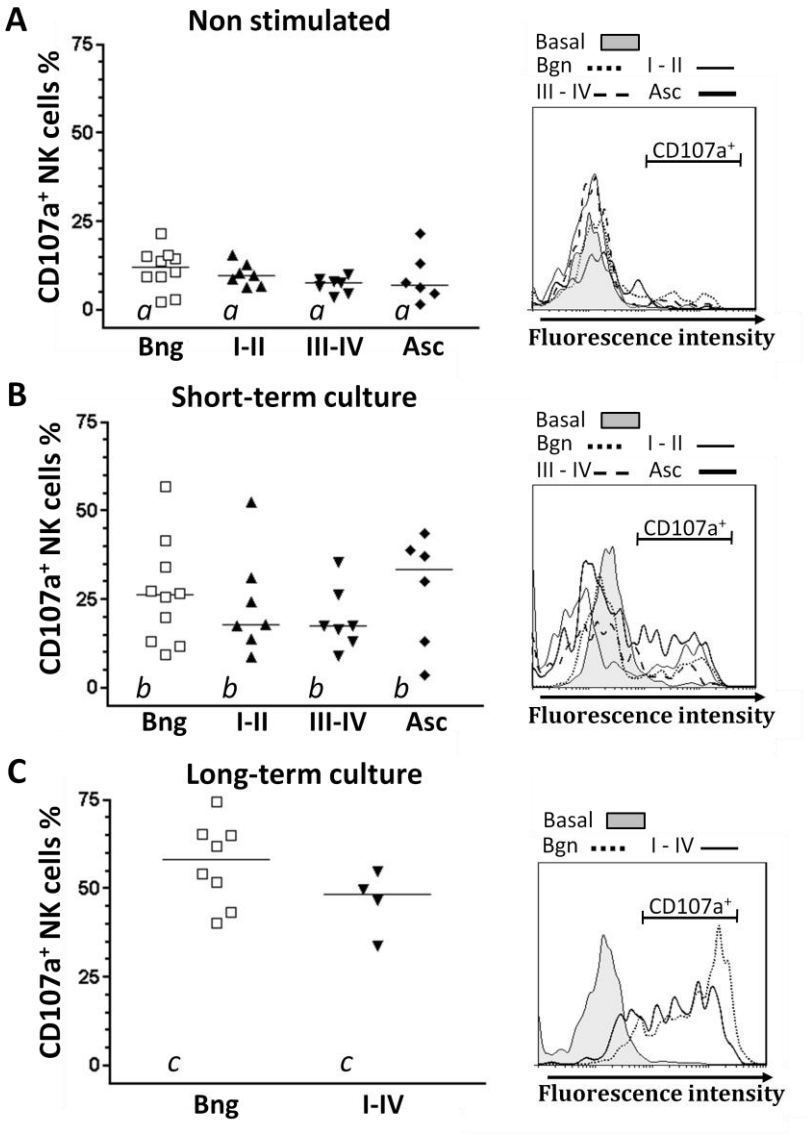

Figure 1. Comparison of functional activation of NK lymphocytes from patients with adnexial alterations (Benign adnexial alteration, Bng; Malignant ovarian neoplasia grade I-II, I-II; Malignant ovarian neoplasia grade II-IV; III-IV and Ascites, Asc). Functional activation were evaluated in NK lymphocytes from blood (A.non and B. short-term IL-2 stimulated and C. long-term cultured) and from ascites (A.non and B. short-term IL-2 stimulated) by the expression of CD107a molecule, while coincubated (ratio 1:1) with K562 target cells. Short-term stimulation with rhIL-2 (1000UI/ml) was conducted overnight in RPMI-1640 medium supplemented with FBS (10%) and L-glutamine (2mM). Long-term culture was conducted by a 21 day procedure with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), rhIL-2 (1000UI/ml) and FBS (10%). Values are presented as median and distribution of frequencies. Groups and treatments (A x B x C) labeled with different letters are significantly different ($p<0.05$). Histograms are representative of the CD107a fluorescence intensity profiles.

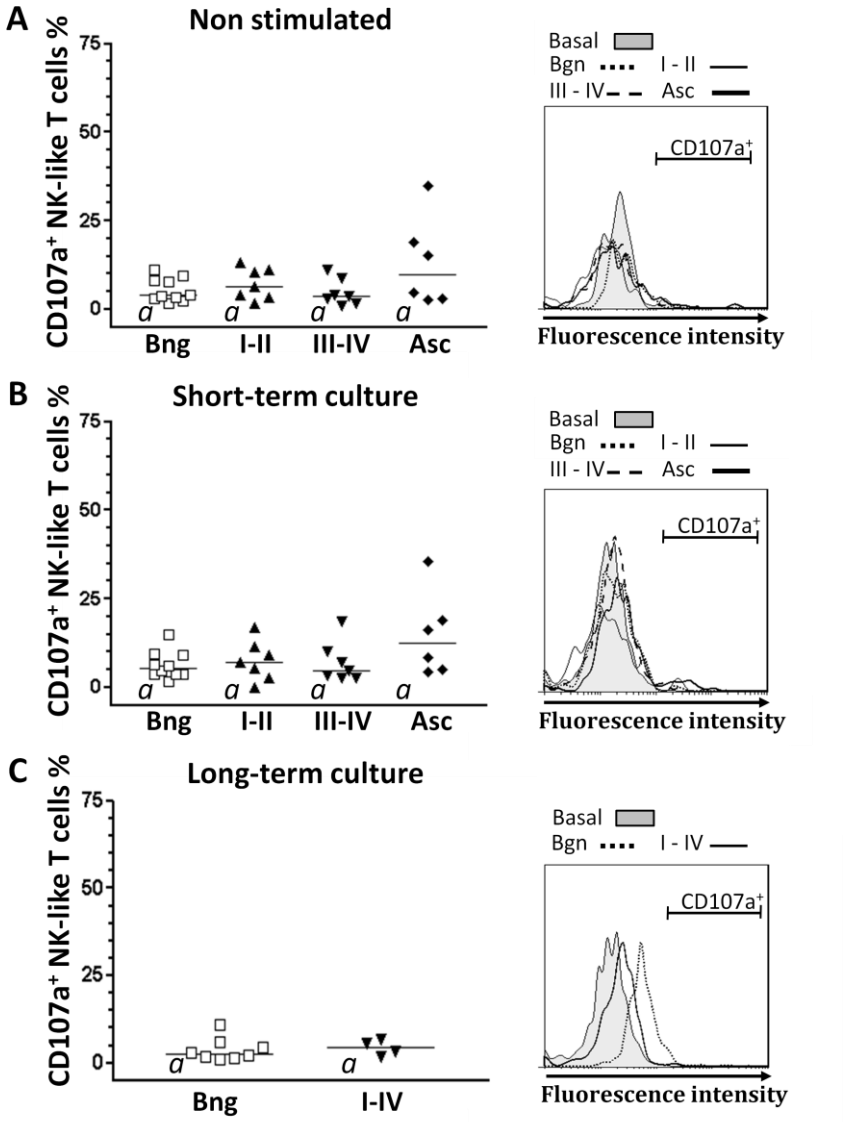

Figure 2. Comparison of functional activation of NK-like T lymphocytes from patients with adnexial alterations (Benign adnexial alteration, Bng; Malignant ovarian neoplasia grade I-II, I-II; Malignant ovarian neoplasia grade II-IV; III-IV and Ascites, Asc). Functional activation were evaluated in NK-like T lymphocutes from blood (A.non and B. short-term IL-2 stimulated and C. long-term cultured) and from ascites (A.non and B. short-term IL-2 stimulated) by the expression of CD107a molecule, while coincubated (ratio 1:1) with K562 target cells. Short-term stimulation with rhIL-2 (1000UI/ml) was conducted overnight in RPMI-1640 medium supplemented with FBS (10%) and L-glutamine (2mM). Long-term culture was conducted by a 21 day procedure with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), rhIL-2 (1000UI/ml) and FBS (10%). Values are presented as median and distribution of frequencies. Groups and treatments (A x B x C) labeled with different letters are significantly different ($p<0.05$). Histograms are representative of the CD107a fluorescence intensity profiles.

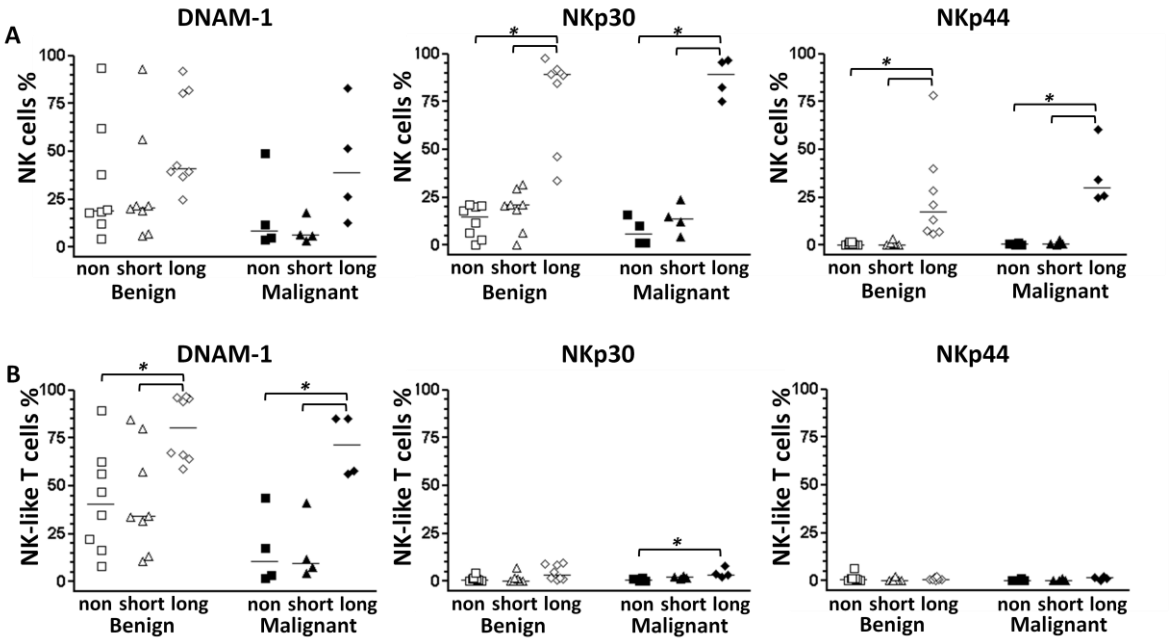

Figure 3. Comparison of the activating receptor expression (DNAM-1, NKp30 and NKp44) on NK (A) and NK-like T (B) lymphocytes from blood of patients with adnexial benign alterations (Benig, n=8) and malignant ovarian neoplasia (Malignant, n=4) non IL-2 stimulated (non), short-term IL-2 stimulated (short) and long-term culture (long). Short-term stimulation of PBMS with rhIL-2 (1000UI/ml) was conducted overnight in RPMI-1640 medium supplemented with FBS (10%) and L-glutamine (2mM). Long-term cultured of PBMC was conducted by a 21 day culturing process with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), rhIL-2 (1000UI/ml) and FBS (10%). Values are presented as median and distribution of frequencies Treatments labeled with ***** are significantly different ($p<0.05$).

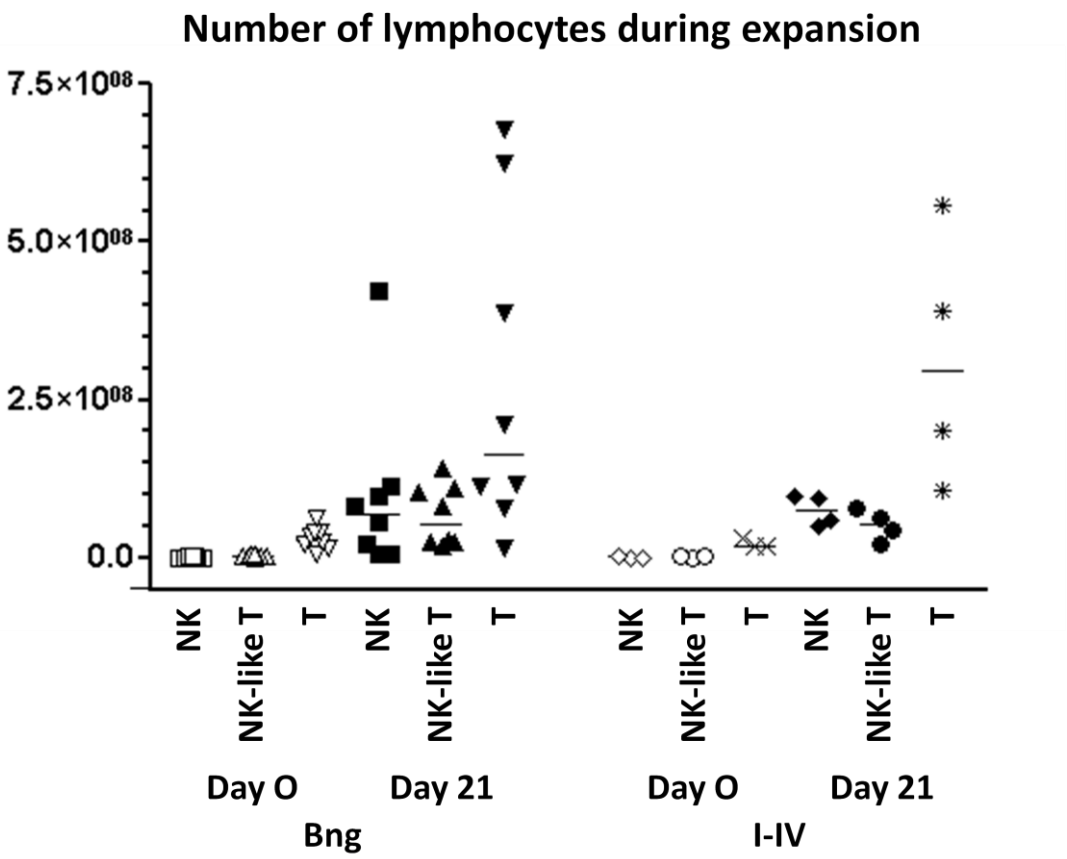

Figure 4. Absolute number of lymphocyte subtypes (NK, NK-like T and T) throughout the long-term culture of PBMC (Day 0=1x10^6 total cells) from patients with adnexial benign alterations (Bng, n=8) and malignant ovarian neoplasia (I-IV, n=4). Long-term stimulation of PBMC was conducted by a 21 day culturing process with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), rhIL-2 (1000UI/ml) and FBS (10%).Values are presented as median and distribution of absolute numbers.

Conclusão

5. CONCLUSÃO

O sistema de cultura de longo-duração de CMSP de pacientes com neoplasia de ovário resultou em elevada porcentagem de células NK funcionais.

O sistema de cultura de longo-duração promoveu o aumento da expressão dos receptores ativadores DNAM-1 e NKp30 nas células NK, representando um meio para a restauração da expressão do DNAM-1, frequentemente suprimido em pacientes com carcinoma de ovário.

A baixa porcentagem de degranulação das células NK-*like* T sugere que, no sistema experimental aplicado, estas células não são ativadas pela via imune inata.

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Anexos

7. ANEXOS

7.1 Anexo 1 *Termo de Consentimento Livre e Esclarecido*

Termo de Consentimento Livre e Esclarecido

Projeto: **Potencial terapêutico das células NK expandidas *ex vivo* no tratamento do câncer de ovário**

Pesquisador responsável: **Rodrigo Silva**

Objetivo da pesquisa:

Fui informada que o objetivo deste trabalho é o de estudar se as células "natural killer" (células de defesa presentes no sangue e no líquido ascítico coletados) conseguem destruir células do câncer de ovário. Para que este estudo seja realizado as células "natural killer" serão submetidas a um processo laboratorial que resultará no aumento do número destas células.

Descrição dos procedimentos:

Minha participação neste estudo é voluntária, sem nenhum ganho financeiro por participar.

Não existem riscos para a minha saúde se eu doar sangue ou líquido ascítico para realização do estudo.

A quantidade de sangue doado será de aproximadamente 20ml.

Este material será usado e parte será guardado para exames especiais e estudos futuros.

O sangue e o líquido ascítico coletados serão utilizados apenas no laboratório para o estudo.

O sangue coletado não será usado em programas de doação de células, tecidos ou órgãos.

O sangue coletado, bem como, células ou produtos derivados da amostra de sangue ou de ascite não serão comercializados em nenhuma situação.

Benefícios:

Minha participação no estudo é voluntária sem nenhum benefício imediato. Entretanto, os resultados obtidos com esta pesquisa poderão ajudar no entendimento da resposta imune contra o câncer e auxiliar na elaboração de novos tratamentos para esta doença.

Transtornos ou riscos:

Fui informada que não estão previstos transtornos ou riscos, a não serem os riscos comuns inerentes da colheita de sangue ou drenagem do líquido ascítico.

Confidencialidade e Privacidade:

Todas as informações sobre minha pessoa serão mantidas em segredo e minha privacidade será mantida. Os resultados da pesquisa serão mantidos em sigilo pelos pesquisadores e, mais tarde, publicados sem que minha identidade seja revelada.

Em qualquer momento, em caso de dúvida ou reclamações poderei procurar o Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP (Telefone: 3521-8936), ou o pesquisador responsável: Rodrigo Silva (Telefone:3521-9462).

Declaro que li as informações neste documento e concordo em participar da pesquisa

Nome:.....RG.....Data:.....

Endereço:.....Telefone:.....

_____ _____

Assinatura da doadora Assinatura do pesquisador

*7.2 **Anexo 2** Estadiamento do carcinoma de ovário 2000 (FIGO)*

FIGO	CARACTERÍSTICAS
O	Sem evidência de tumor primário
I	Tumor confinado aos ovários
IA	Tumor limitado a um ovário, cápsula intacta, **sem** tumor na superfície ovariana, **sem** citologia positiva (células malignas) em líquido ascítico ou lavado peritoneal
IB	Tumor limitado a ambos os ovários, cápsula intacta, **sem** tumor na superfície ovariana, **sem** citologia positiva (células malignas) em líquido ascítico ou lavado peritoneal
IC	Tumor limitado a um ou ambos os ovários, **com** qualquer um dos achados: ruptura de cápsula, tumor na superfície ovariana, citologia positiva (células malignas) em líquido ascítico ou lavado peritoneal
II	Tumor envolvendo um ou ambos os ovários **com** extensão pélvica
IIA	Extensão e/ou implantes no útero e/ou implantes nas tubas uterinas, **sem** citologia positiva (células malignas) em líquido ascítico ou lavado peritoneal
IIB	Extensão para outros órgãos pélvicos, **sem** citologia positiva (células malignas) em líquido ascítico ou lavado peritoneal
IIC	Extensão e/ou implantes no útero e/ou implantes nas tubas uterinas e/ou implantes em outros órgãos pélvicos, **com** citologia positiva (células malignas) em líquido ascítico ou lavado peritoneal
III	Tumor envolvendo um ou ambos os ovários com microscopia confirmando metástase peritoneal além da pélvis e/ou linfonodo regional
IIIA	Microscopia confirmando metástase além da pélvis
IIIB	Metástase peritoneal macroscópica além da pélvis, 2cm ou menos em sua maior dimensão
IIIC	Metástase peritoneal além da pélvis, com mais de 2cm em sua maior dimensão e/ou metástase em linfonodo regional
IV	Metástase além da cavidade peritoneal

Nota: Metástase em região de cápsula hepática (estádio 3), em região de parênquima hepático (estádio 4). Efusão pleural deve ter citologia positiva.

7.3 Anexo 3 Características histopatológicas dos sujeitos

Anexo 3. Características dos sujeitos incluídos nos anos de 2011 e 2012.

Código Paciente	Idade	Estádio Clínico (FIGO)	Diagnóstico Histopatológico
19	68		Cistos foliculares de ovário
20	48		Fibroma
21	53	IIB	Adenocarcinoma seroso pouco diferenciado
22	45		Cistoadenoma mucinoso
23	45	IIIC	Adenocarcinoma seroso papilífero
24	60	IIIC	Adenocarcinoma seroso bem diferenciado
27	65	IIIC	Adenocarcinoma seroso moderadamente diferenciado
28	71		Cistoadenoma seroso
29	43		Cistos foliculares de ovário
30	59	IIIC	Adenocarcinoma seroso de alto grau
31	79	IIIC	Adenocarcinoma mucinoso moderamentediferenciado
32	43		Cistoadenoma seroso papilífero
33	62		Teratoma maduro monodérmico do tipo STRUMA OVARII
34	81	IC	Adenocarcinoma seroso papilifero pouco diferenciado
35	48	IA	Adenocarcinoma edometrióide bem diferenciado
38	47		Cistoadenoma mucinoso
39	79		Cistoadenoma mucinoso
40	47	IIIC	Adenocarcinoma seroso de alto grau
41	54	IIA	Adenocarcinoma seroso papilífero de alto grau
42	45		ND
43	53	IA	Tumor sero-mucinoso papilífero borderline
44	25	IA	Tumor seroso papilífero borderline
45	76	IV	Adenocarcinoma mucinoso bem diferenciado
48	77	IA	Adenocarcinoma seroso papilífero moderadamente diferenciado

Todos os laudos com diagnóstico histopatológico foram emitidos pelo Laboratório de anatomia patológica do Hospital das Clínicas da Universidade de Campinas. ND: não determinado; tumor benigno sem diagnóstico histopatológico.

7.4 Anexo 4 *Percentual dos subtipos de linfócitos (NK, NK-like T, T e B) nas culturas de PBMC e concentrações séricas dos marcadores tumorais CEA e CA125 das pacientes com alterações anexiais de ovário*

-Percentual dos subtipos de linfócitos (NK, NK-*like* T, T e B) nos dias 0, 10 e 21 de cultura das CMSP de pacientes com alterações anexiais de ovário. Os valores estão apresentados como média ± desvio padrão.

Subtipos de linfócitos	**Benigno**			**Maligno**		
	Dia 0 **n=8**	**Dia 10** **n=8**	**Dia 21** **n=8**	**Dia 0** **n=4**	**Dia 10** **n=4**	**Dia 21** **n=4**
NK ($CD3^-CD56^+$)	10,7 ± 8,7	3,6 ± 2,5	20,3 ± 18,7	12,8 ± 10,2	2,7 ± 1,0	20,3 ± 10,4
NK-T ($CD3^+CD56^+$)	3,8 ± 2,8	10,6 ± 10,2	21,0 ± 16,9	4,2 ± 2,6	4,7 ± 4,1	11,6 ± 2,0
T ($CD3^+CD56^-$)	72,0 ± 11,0	83,6 ± 12,4	55,9 ± 20,1	67,1 ± 8,2	91,2 ± 5,3	66,0 ± 12,0
B ($CD3^-CD56^-$)	13,5 ± 6,7	2,2 ± 0,9	2,9 ± 3,2	15,9 ± 4,1	1,4 ± 0,5	2,0 ± 1,0

-Mediana e variação das concentrações séricas dos marcadores tumorais CEA e CA125 de pacientes com neoplasia de ovário. CEA acima de 5ng/ml e CA125 acima de 30U/ml são considerados elevados.

Variáveis Mediana (Variação)	***Benigno (n=10)***	***Maligno sem Metástase (n=7)***	***Maligno com Metástase (n=7)***
CEA (ng/ml)	1,5 (0,83-9,9)	1,3 (0,85-6,7)	1,7 (0,78-2003)
CA125 (U/ml)	28,3 (5,2–1520)	96,1 (5,2–1682)	358,0 (27,6–3954)

7.5 Anexo 5 *Percentual dos subtipos de linfócitos (NK, NK-like T, T e B)* presentes sangue periférico e na ascite de pacientes com alterações anexiais de ovário.

-Percentual dos subtipos de linfócitos (NK, NK-*like* T, T e B) presentes no sangue periférico e na ascite de pacientes com alterações anexiais de ovário. As amostras de sangue das pacientes foram agrupadas segundo as características das alterações anexiais (benigno e maligno) e estadiamento, resultando nos grupos benigno, maligno (I-II) e maligno (III-IV). stim: linfócitos estimulados com IL-2 (1000UI/106cel/ml) por 16-18h a 37˚C; n-stim: linfócitos não estimulados. Os valores estão apresentados como média ± desvio padrão.

Subtipos de linfócitos	**Benigno**	**Benigno**	**Maligno (I-II)**	**Maligno (I-II)**	**Maligno (III-IV)**	**Maligno (III-IV)**	**Ascite**	**Ascite**
	n-stim	**stim**	**n-stim**	**stim**	**n-stim**	**stim**	**n-stim**	**stim**
	n=10	**n=10**	**n=7**	**n=7**	**n=7**	**n=7**	**n=6**	**n=6**
NK (CD3-CD56+)	9,3 ± 8,3	9,5 ± 8,1	12,0 ± 10,3	11,7 ± 9,2	14,3 ± 8,0	14,6 ± 8,2	7,5 ± 3,7	8,8 ± 4,5
NK-T (CD3+CD56+)	4,2 ± 2,6	3,8 ± 1,8	5,4 ± 5,5	5,7 ± 5,9	6,0 ± 6,0	6,3 ± 6,3	3,8 ± 2,7	4,7 ± 4,0
T (CD3+CD56-)	72,9 ± 10,0	73,4 ± 9,2	64,9±10,6	64,3 ± 9,6	55,8 ± 16,1	55,2 ± 15,6	72,2 ± 10,9	70,8 ± 11,1
B (CD19+)	13,6 ± 6,0	13,3 ± 5,6	17,7 ± 9,1	18,4 ± 9,3	23,8 ± 14,8	23,9 ± 15,9	16,6 ± 9,8	15,6 ± 8,6

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NK Cell-Mediated Targeting of Human Ovarian Cancer and Possibilities for Immunotherapy

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Abstract

Silva RF, Alves PCM, Guimarães F. NK Cell-Mediated Targeting of Human Ovarian Cancer and Possibilities for Immunotherapy. ARBS Annu Rev Biomed Sci 2011;13:23-29. A better understanding of the NK cell receptor-ligand interaction opened possibilities for new therapeutic strategies. The well known mismatch KIR/HLA interaction is of great importance for NK cell stimulation. Lately, the interaction of the activating receptor DNAM-1 with its ligand PVR, has been highlighted in many ovarian carcinoma studies. Autologous and allogeneic NK cells, along with monoclonal antibodies, have been tested in current immunotherapies. This review presents information on NK cell receptor-ligand interactions and its possibilities for NK-based immunotherapy against ovarian cancer.

Keywords: Natural killer cells, immunotherapy, ovarian neoplasm, NK cell receptors.

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1. Introduction

The poor prognosis of ovarian carcinoma has driven studies to develop more efficient therapeutic strategies. The possibility to use natural killer (NK) cells for the treatment of human cancer has increased recently (Ljunggren & Malmberg, 2007; Sutlu & Alici, 2009). This is a consequence of an increased comprehension of the molecular processes and receptor-ligand interactions which influences NK cell

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recognition and elimination of tumor cells. New cellular isolation and expansion methods ex vivo provide sufficient quantities of human NK cells for clinical trials (Barkholt *et al.*, 2009). Additionally, drugs, antibodies and genetic manipulation can be combined to the therapeutic strategy in order to exploit NK cells antitumor function (Malmberg *et al.*, 2008). The purpose of this review is to present information on NK cell specific molecular receptor-ligand interactions with ovarian cancer cells and its usefulness for the development of an NK-based immunotheraphy.

2. NK Cells

NK cells are lymphocytes initially indentified by its functionality, based on their capacity of eliminating a variety of tumor cells without previous stimulation (Kiessling *et al.*, 1975; Herbermen *et al.*, 1975). In humans, NK cells constitute 5-15% of lymphocytes present in the peripheral blood, identified by the absence of CD3 cell surface molecule and by the expression of CD56 (CD3-CD56+), an isoform of neural cell adhesion molecule (NCAM) also found on a minority of T cells (Robertson *et al.*, 1990; Arnon *et al.*, 2006). NK cells can be further grouped into two functional subtypes considering the expression of the CD56 marker. The majority (90%) of the NK cells that are found in the peripheral blood express low maker density, they are categorized as CD56dim and the minority of them (10%) express high marker density, they are categorized as CD56bright (Lanier *et al.*, 1986; Sutlu & Alici, 2009). CD56dim cells can be found in the bone marrow, peripheral blood and spleen. They express high levels of CD16 (FcγRIIIA) receptors, have great cytolytic potential and produce less variety of cytokines. CD56bright are mainly found in peripheral lymphoid tissues, express low levels of CD16, have low cytolytic capacity and produce the largest variety of immunoregulatory cytokines. It has been hypothesized that CD56bright cells are less mature than CD56dim, since there is an increase in the expression of specific receptors of the subtype "dim" (like activation receptors and CD16+) when the "bright" subtype is stimulated by IL-2 (Lanier *et al.*, 1986; Di Santo, 2006). Another evidence which supports this hypothesis is the existence of large quantities of CD56bright cells associated with lymphocytes CD34+CD45RA+, NK precursor cells in secondary lymphoid tissues. In these tissues there are still plenty of antigen presenting cells expressing interleukin 15 (IL-15) attached to its surface, this cytokine is known to be important in the maturation of NK cells. (Mattei *et al.*, 2001; Fehniger *et al.*, 2003; Freud *et al.*, 2005; Caligiuri, 2008). Therefore, the development of NK cells does not only occur in the bone marrow, but it also occurs in secondary lymphoid tissues.

NK cells are mentioned as lymphocytes of the innate immune response, mediating effector functions against malignant or virus infected cells. However, NK cells production of pro-inflammatory cytokines such as interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α) and granulocyte macrophage colony-stimulation factor (GM-CSF) regulates the innate immune response and contributes to the development of the adaptive immune response (Wiltrout, 2000). The cytotoxic activity of NK cells occurs after contact with target cells, followed by the secretion of perforin and granzyme-B or induction of apoptosis by ligands FasL and TRAIL (TNF-related apoptosis-inducing ligand) (Wiltrout, 2000; Cooper *et al.*, 2001; Wu & Lanier, 2003; French & Yokoyama, 2003; Hayakawa *et al.*, 2004).

3. NK Receptors

NK cells cytolytic function is determined by the balance of inhibitory and activating signals resulted from the interaction between NK cells receptors and target cells ligands (Cerwenka *et al.*, 2001; Lanier, 2005). Several activating and inhibitory receptors have been described in NK cells, some of them have been characterized focusing on the recognition process of ovarian cancer cells. Among the major inhibitory receptors are: KIR (killer immunoglubuline-like receptors) that recognize ligand molecules of the human leukocyte antigen (HLA) class I from the groups A, B and C; CD94-NKG2A receptor that recognizes the HLA class I from the group E; and ILT2 (immunoglobulin-like transcript 2) receptor that recognizes a relatively conserved region in the class I molecule, providing broad class I specificity (Farag *et al.*, 2002; Moretta *et al.*, 2004; Lanier, 2005; Malmberg *et al.*, 2008). HLA class I is expressed by virtually all nucleated cells of a person, playing a key role in the recognition of body cells as "self" by the immune system. Once committed to HLA class I "self", the inhibitory receptors KIR signal with dominance over the signal of activating receptors, therefore hindering the cytolytic function of NK cells (Bryceson *et al.*, 2006; Caligiuri, 2008). Thus, NK cells spare autosomal cells that express normal levels of HLA class I molecules while eliminating abnormal cells, such as tumor cells or cells infected by viruses, which frequently lose their expression of HLA class I (Ljunggren *et al.*, 1990; Garcia-Lora *et al.*, 2003; Schanoski *et al.*, 2004).

Among the activating receptors stand out the natural cytotoxic receptors (NCR) NKp46 and NKp30

that are expressed constitutively in NK cells, and the NKp44 receptor that is expressed after NK cells activation by interleukin 2 (IL-2) (Arnon *et al.*, 2006; Fuchs *et al.*, 2005). These receptors also assist in the recognition of tumor cells, cells infected by viruses and maturation regulation of dendritic cells. However, little is known about the identity of the ligands for NCR (Cooper & Caligiuri, 2004; Arnon *et al.*, 2005). The density of NCR expression varies among individuals and correlates directly with the ability of NK cells to eliminate abnormal cells (Sivori *et al.*, 1999; Pende *et al.*, 2001). Unlike the NCR, the NKG2D is an activating receptor whose expression is not restricted to NK cells, it is also expressed by cytotoxic T lymphocytes (γ/δ T e α/β T-CD8). The NKG2D recognizes as ligands molecules homologous to HLA class I, represented by transmembrane proteins such as MIC/A, MIC/B, ULBP4 (UL16-biding protein) and proteins anchored to the cell surface by glicofosfatidilinositol (GPI) as ULBP 1, 2 and 3 (Bauer *et al.*, 1999; Biassoni *et al.*, 2001; Cosman *et al.*, 2001; Raulet, 2003; Coudert & Held, 2006). In humans, increased expression of MIC and ULBP is related to different forms of cellular stress, such as viral infection and malignant transformation (Bauer *et al.*, 1999; Cosman *et al.*, 2001; Onda *et al.*, 2001). In fact, the expression of MICA/B has been observed in most human epithelial tumors, including breast, ovarian, colon, kidney and lung carcinomas (Biassoni *et al.*, 2001; Diefenbach & Raulet, 2002), contributing to the possible susceptibility of these tumors to the cytotoxic activity of NK cells. DNAX accessory molecule-1 (DNAM-1) is another important NK cells activating receptor. The ligands indentified for DNAM-1 receptor are the Poliovirus receptor (CD155) and Nectin-2 (CD112), with CD155 displaying to have a predominant role in the induction of DNAM-1 responses. Furthermore, CD155 is commonly expressed on normal cells and overexpressed on various tumor types, including ovarian carcinoma (Lanier, 2005; Carlsten *et al.*, 2009). DNAM-1 contributes to tumor immune surveillance and plays a crucial role in NK cell-mediated recognition of several types of human tumors.

4. NK Cell in Response Against Ovarian Cancer

Since NK cells were first described, more than 30 years ago, new cancer therapies based on their capacity to lysis tumor cells have been developed. Although, several studies have demonstrated the ability of NK cells to target tumor cells in vitro and in vivo (Smyth *et al.*, 2002; Wu & Lanier 2003; Malmberg *et al.* 2008), only in the last decade have been obtained direct evidences on how receptor-ligand interactions drive targeting of tumor cells by NK cells in humans. This information has prompted new insights on therapeutic uses for NK cells.

NK cells from ovarian carcinoma patients were initially reported to display none or poor cytolitic activity against ovarian cell lines, fresh ovarian tumors, and even against the prototype NK cell target K562 (Lotzova *et al.*, 1986; Lotzova *et al.*, 1988; Roszkowski *et al.*, 1993; Malberg, 2004). Additionally, NK cell cytolitic activity against tumor cells was significantly lower among patients with ovarian carcinoma than in patients with benign masses (Lutgendorf *et al.*, 2005). However, antitumor function of NK cells from patients with ovarian cancer can be reestablished as demonstrated by in vitro stimulation of effector cells with recombinant IL-2 (Lotzova *et al.*, 1986; Lotzova *et al.*, 1988) or by enriching the preparation of effector cells with large granular lymphocyte, corresponding to the NK cells (Lotzova *et al.*, 1988). These observations support the idea of overcoming the immunosuppression often seen in patients with cancer, by using strategies for ex vivo expansion and stimulation of cytotoxic cells.

Similarly to other malignancies, ovarian cancer exploits an array of immunological ways to create a suppressive environment to prevent being eliminated by the immune response (Yigit *et al.*, 2010). Two immunosupressive mechanisms capable of affecting NK cell functions have already been detected in ovarian carcinoma patients, one involving recruitment of regulatory T CD4+CD25+ (Treg) lymphocytes to the tumor site, and other involving selective down-regulation of NK cell-activating receptor DNAM-1 (Curiel *et al.*, 2004; Yigit *et al.*, 2010; Carlsten *et al.*, 2009). Specific recruitment of Treg lymphocytes to the tumor site and ascites was correlated to a reduced survival of patients with ovarian cancer (Curiel *et al.*, 2004). Additionally, Treg lymphocytes have been reported to affect NK cell proliferation, cytotoxicity and IFN-γ production (Ghiringhelli *et al.*, 2005; Smyth *et al.*, 2006). Ovarian carcinoma cells expressing the DNAM-1 ligand CD155 led to down-regulation of DNAM-1 activating receptor, explaining the hyporesponsiveness found in tumor-associated NK cells compared to the autologous peripheral blood NK cells (Carlsten *et al.*, 2009).

Immunosuppression is not advantageous for immunotherapies, but by knowing the mechanisms of the suppression, new strategies can be developed in an attempt to overcome this therapeutic barrier. Recent studies have demonstrated the possibility of generating CD56+ NK and NKT-like lymphocytes by ex vivo expansion of PBMC (peripheral blood mononuclear cells) from patients with ovarian carcinoma (Alves *et*

al., 2011). Such effector cell preparations displayed antitumor function, showing the feasibility of overcoming the immune impairment often inferred to cancer patients. Additionally, the NK cells present in the ex vivo effector cells expansion were CD16+, indicating their activation status and their cytotoxic potential mediated by antibodies (Borghaei *et al.*, 2009).

Allogeneic human NK cells are also known to recognize and kill freshly isolated ovarian carcinoma cells. The degranulation of NK cells is dependent on signaling through DNAM-1 receptors with an additional contribution of NKG2D receptors by recognizing corresponding ligands expressed on the surface of ovarian carcinoma cells (Carlsten *et al.*, 2007). The relative high expression of CD155 in combination with reduced levels of HLA class 1 molecules on ovarian carcinoma cells, labels them as an ideal target for autologous NK cells as well (Carlsten et al 2009). Allogeneic NK cells from healthy donors can be a promising immunotherapy strategy since they don't exhibit impaired functions consequently derived from induced immune suppression. Besides, allogeneic NK cells can be specifically activated by the mismatch interaction of KIR/HLA and by the positive interaction between the activating receptor DNAM-1 and its ligand PVR (Carlsten *et al.*, 2007). Graft-versus-host disease (GVHD) is of major concern in the usage of allogeneic NK cells for immunotherapy. However, many recent clinical trials have demonstrated no GVHD in patients with malignancies treated with allogeneic NK cells (Passweg *et al.*, 2004; Miller *et al.*, 2005; Barkholt *et al.*, 2009).

Monoclonal antibodies (mAbs) usage in cancer therapy is based on targeting tumor cells that express tumor associated antigens. Many mAbs targeting antigens associated to cancer cells have been developed in the past few years and it is lately, one of the most important drugs approved for the treatment of cancer, including ovarian carcinoma (McCall *et al.*, 2001; Chan *et al.*, 2006; Seimetz *et al.*, 2010; Esser *et al.*, 2011). The efficacy of mAbs on hematological and some solid malignancies has been shown, but in the case of ovarian carcinoma, it has not been yet validated (Mabuchi *et al.*, 2010). Several mAbs have been investigated for a potential treatment against ovarian cancer, such as bevacizumab; a vascular endothelial growth factor-targeted mAb therapy, trastuzumab and cetuximab; an epidermal growth factor-targeted mAb therapy, oregovomab; a CA-125-targeted mAb therapy, the mAb human milk fat globule 1 (HMFG1); a MUC1-targeted therapy and catumaxomab; a trifunctional antibody with two different antigen-binding specificities, epithelial cell adhesion molecule (EpCAM) and CD3 antigen (Seimetz *et al.*, 2010; Mabuchi *et al.*, 2010). Their efficacy and side effects have been demonstrated in clinical trials but still, further research is needed to create more efficient, precise and less toxic immunotherapy strategies for ovarian carcinoma (Mabuchi *et al.*, 2010). Combined therapies using mAbs with NK cells are believed to benefit from antibody-dependent cell-mediated cytotoxicity (ADCC), since NK cells express CD16 (FcγRIIIA) receptor that recognizes the Fc portion of mAbs (McCall *et al.*, 2001; Borghaei *et al.*, 2009).

5. Conclusion

Due to an increased knowledge of the molecular processes and receptor-ligand interactions of NK cells, studies have been conducted using these lymphocytes for the development of cancer treatments. Currently, clinical trials with sufficient amount of NK cells can be conducted, due to improvements on the isolation and expansion methods. In overall, autologous and allogeneic NK cells, the KIR/HLA mismatch, DNAM-1/PVR interaction and monoclonal antibodies, currently are the most promising factors for NK-based immunotherapy against ovarian cancer.

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7.7 Anexo 7 **Resumo publicado e apresentado no XXXVII Congress of the Brazilian Society of Immunology 2012**

INNATE ACTIVATION OF NK AND NK-LIKE T LYMPHOCYTES FROM OVARIAN CANCER PATIENTS.

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Introduction: NK lymphocytes are known by their ability to eliminate a variety of malignant cells in a process involving innate recognition by an array of stimulatory and inhibitory receptors. Similarly, the variant subset of NK-like T lymphocytes has been reported to eliminate tumor cells, but the targeting process might involve either innate or adaptive immune recognition. This study aimed to evaluate the functional activation of NK and NK-like T cells (pre, short- and long-term IL-2 stimulated), from blood and ascites of ovarian neoplasia patients.

Methods and Results: Blood was collected from 20 patients with pelvic neoplasias after signed consent: 9 benign (Bng, 6 ovarian neoplasia and 3 other pelvic neoplasia), 5 ovarian malignant without metastasis (Mlg) and 6 ovarian malignant with metastasis (MlgMt). Ascites (Asc) was collected from 6 patients with ovarian neoplasia. Mononuclear cells were separated by Ficoll-Paque gradient. NK and NK-like T cells activation (pre, short- and long-term stimulated) were evaluated against K562 (1:1 ratio) by the expression of CD107a, analyzed by flow cytometry. Short-term stimulation with IL-2 (1000UI/ml) was conducted overnight in RPMI-1640 medium supplemented with FBS (10%) and L-glutamine (2mM). Long-term stimulation was conducted by a 21 day culturing process with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), IL-2 (1000UI/ml) and FBS (10%). NK functional activation of pre stimulated cells, seemed to be impaired by the development of the disease as assessed by the MFI median/variation (Bng=72.90/ 0.54-134.09; Mlg=53.32/ 29.63-74.54; MlgMt=25.83/ 10.99-59.13; Asc=15.17/ 3.50-99.52). Short term stimulation increased significantly ($p<0.05$) NK cells activation (Bng=133.18/ 7.25-441.15; Mlg=239.53/ 122.62-367.17; MlgMt=127.46/ 59.24-241.40; Asc=156.77/ 11.41-241.35). NK-like T cells showed no activation and short-term IL-2 stimulation caused any effect. Interestingly, long-term stimulation elicited NK-like T functional activation. Statistical analysis inter groups was performed by Kruskal-Wallis test and intra groups by Mann Whitney test.

Conclusion: Results indicate that the functional integrity of NK cells is impaired as ovarian malignancies develop. Short-term stimulation restored functional activation of NK cells from patients of malignant groups. NK-like T lymphocytes were activated by innate recognition only after long-term stimulation.

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7.8 Anexo 8 *Resumo aceito no 15th International Congress of Immunology 2013 e para publicação no Frontiers in Immunology*

IMPROVEMENT OF NK CELLS FUNCTIONAL ACTIVATION AFTER LONG TERM IL-2 STIMULATION *IN VITRO* FROM OVARIAN NEOPLASIA PATIENTS

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Introduction: NK cells are lymphocytes known by their ability to eliminate a variety of malignant cells without previous stimulation, in a process involving innate recognition by an array of stimulatory and inhibitory receptors. Similarly, the variant subset of NKlike T lymphocytes has been reported to eliminate tumor cells, but the targeting process might involve either innate or adaptive immune recognition. This study evaluated the functional activation of NK and NK-like T cells, the expression of activating receptors DNAM-1, NKp30 and NKp44 (pre, short- and long-term IL-2 stimulated), from blood and ascites of ovarian neoplasia patients.
Methods and Results: Blood and ascites were collected from 24 patients with ovarian neoplasias after signed consent: 11 benign (Bng), 6 malignant without metastasis (Mlg) and 7 malignant with metastasis (MlgMt). Ascites (Asc) was collected from 6 patients with ovarian neoplasia. Mononuclear cells were separated by Ficoll-Paque gradient. NK and NK-like T cells activation (pre, short- and long-term stimulated) were evaluated against K562 (1:1 ratio) by the expression of CD107a, analyzed by flow cytometry. Short-term stimulation with IL-2 (1000UI/ml) was conducted overnight in RPMI-1640 medium supplemented with FBS (10%) and L-glutamine (2mM). Long-term stimulation was conducted by a 21 day culturing process with SCGM CellGro medium supplemented with anti-CD3 (10ng/ml, first 5 days), IL-2 (1000UI/ml) and FBS (10%). NK functional activation of pre stimulated cells, seemed to be impaired as the disease develops, as assessed by the percentage median/variation (Bng=13.86/ 2.25-21.78; Mlg=9.57/ 6.48-12.96; MlgMt=7.73/ 3.65-10.06; Asc=6.99/ 1.68-21.68). Short-term stimulation increased NK cells activation (Bng=24.19/ 9.52-57.03; Mlg=24.49/ 8.69-52.37; MlgMt=17.59/ 9.28-35.63; Asc=33.54/ 3.83-43.50). Long-term stimulation increased NK cells activation significantly ($p<0.001$) (Bng=58.04/ 40.39-74.48; Mlg=46.61/ 33.89-54.76). The percentage of NK cells expressing the activating receptors DNAM-1, NKp30 and NKp44 increased significantly ($p<0.05$) after long-term stimulation. NK-like T cells showed no activation on pre, short- and long-term IL-2 stimulation. Statistical analysis inter groups was performed by Kruskal-Wallis test and intra groups by Mann Whitney test.
Conclusion: The functional integrity of NK cells was impaired as ovarian malignancies develop. Long-term stimulation resulted in a much higher number of functional NK cells compared to short-term, entitling this method for adoptive therapy. Long-term stimulation was particularly efficient to up-regulate DNAM-1 activating receptor on NK cells, representing a way to overcome down-regulation demonstrated on patients with ovarian carcinoma. The lack of activation of NK-like T cells (pre, short- and long-term IL-2 stimulated) suggests that these cells are not activated through innate pathway but through adaptive pathway.
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